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THE UNIVERSITY OF ALBERTA

DC POLAROGRAPHIC ASSAY OF CERTAIN TETRACYCLINE ANTIBIOTICS

BY

© KUN-SHIH HUANG, B.Sc.

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "DC Polarographic Assay of Certain Tetracycline Antibiotics" submitted by Kun-shih Huang in partial fulfilment of the requirements for the degree of Master of Science.

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TO

MY PARENTS

ABSTRACT

The electroreduction of certain tetracyclines at the dropping mercury electrode (DME) has been used as the basis for a quantitative analytical procedure for these drugs. The total "diffusion" current was measured for this purpose throughout the studies. The electroreduction of tetracycline hydrochlorides was studied according to the following steps: First, different well buffered aqueous solutions were tried and the optimum one was selected, then the optimum pH within the chosen buffer system was determined. It was found that the total polarographic response, the $E_{1/2}$ and the limiting current were all pH dependent. Secondly, by varying the mercury column height the electroreduction process was identified as being partially diffusion dependent and partially due to some kinetic factor. Finally, the application of the logarithmic analysis principle for the determination of the electroreduction mechanism was studied.

For all the tetracyclines investigated, linear and reproducible calibration curves were readily obtained by plotting total current versus concentration. The technique was subsequently applied to a variety of different pharmaceutical dosage forms and in most of the cases, good egreement was observed between the results obtained by the present method and those supplied by the pharmaceutical manufacturers using their procedures.

Owing to the versatility and sensitivity of polarography, preliminary investigations have been made regarding the applicability of this technique to the quantitative analysis of the tetracyclines in biological samples such as urine and blood.

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INTRODUCTION

I. Activity and Structural Similarities of Tetracycline Antibiotics

The tetracyclines are the prototypes of the broad spectrum antibiotics, so-called because they inhibit the growth of a wide range of microorganisms, including many gram-positive and gram-negative bacteria, species of rickettsia and mycoplasma (PPLO), certain protozoa and large viruses.

Since the appearance of the original reports on the tetracyclines, a large number of derivatives, degradation products and similar compounds, having a wide range of antibiotic activity, have been described. The present studies will concern themselves largely with the four antibiotics which are used extensively in the clinic: tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC) and demethylchlortetracycline (DMCTC). The close chemical similarity among these antibiotics, their similar antibiotic spectra and the mutual cross-resistance which often develops to them have led to the general assumption that the mechanisms by which the tetracyclines inhibit the growth of microbial cells are similar, if not identical. Three of the most acceptable assumptions summarized by Goodman and Gilman (1) are that the antibiotics exert their effect by means of:

(i) active chelation of metal cations,

(ii) inhibition of synthesis of specific enzymes,

(iii) suppression of protein synthesis.

It must be realized, however, despite the plausibility of

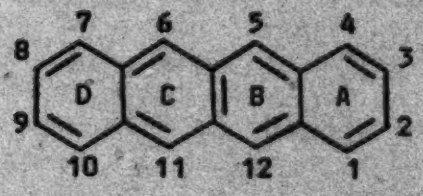
each of these assumptions, that the exact mechanism of action of these drugs is not yet known.

Advantages of the use of tetracyclines as chemotherapeutic agents are their effectiveness, their favorable pharmacodynamics *in vivo*, their low toxicity, their broad-spectrum activity and their oral efficacy.

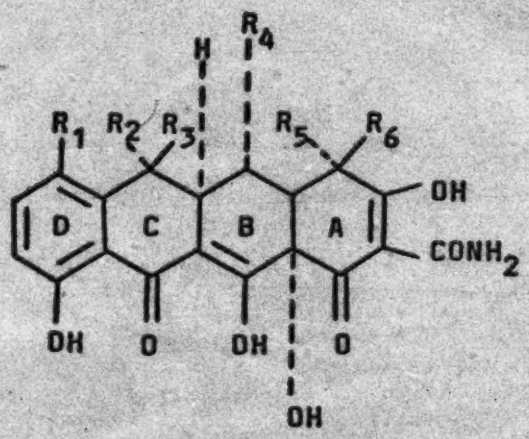
Today the therapeutic importance of tetracyclines in the treatment of human infectious diseases is already well known. Increasingly their applications for treating some animal and plant diseases and their inclusion in animal feeds have rendered them the most popular antibiotics in the field of chemotherapy. Such wide spread utilization of these products natually requires the development of reliable methods for quality control, drug availability studies and other purposes.

Structural Formulas

The tetracyclines actually are naphthacene derivatives with the basic ring structure:



Tetracycline Antibiotics (active forms) Employed in This Study:



Name	R_1	R_2	R_3	R_4	R_5	R_6	M.W. (USP)
TC	H	CH_3	OH	H	$N(CH_3)_2$	H	free base 444.45 HCl salt 480.91 *phosphate complex not known
OTC	H	CH_3	OH	OH	$N(CH_3)_2$	H	free base 460.44 HCl salt 496.91
CTC	Cl	CH_3	OH	H	$N(CH_3)_2$	H	free base 478.89 HCl salt 515.35
DMCTC	Cl	H	OH	H	$N(CH_3)_2$	H	free base 464.86 HCl salt 501.32

*Tetracycline Phosphate Complex (tetracycline sodium hexametaphosphate complex): The precise structural formula of this complex is not known since the complexes vary in size. An approximate empirical formula for tetracycline phosphate complex may be denoted as follows:

$$\left[(C_{22}H_{24}N_2O_8)\cdot Na(PO_3)_6\right]_x\cdot H_2O$$

II. Literature Review

The earliest methods of assaying tetracyclines were the microbiological methods and these are still maintained as the recognized official ones by the USP XVIII, 1970 (2) (A spectrophotometric method for the assay of oxytetracycline·HCl is also given), the BP, 1973 (3), and the NF XIII, 1970 (4). The basis of the microbiological assays is just a simple comparison of inhibition of growth of some selected susceptible microorganism produced by known concentrations of the standard preparations with that produced by the sample preparations being tested. In other words, this method involves a direct measurement of pharmacological activity. Although with proper selection of conditions and microorganisms, microbiological methods are capable of great sensitivity and also a little specificity, at least for a particular class of antibiotics, their reliability is nonetheless somewhat doubtful.

The commonest procedures in the microbiological methods include: (i) Paper Disc Method, (ii) Cylinder-Plate Method and (iii) Turbidimetric Method.

In the paper disc method, circular filter paper discs are soaked in the antibiotic solution and then placed on the agar media contained in a petri dish which has already been seeded with a selected, susceptible test microorganism. The antibiotic thus will diffuse into the media and inhibit the

growth of the microorganism under and surrounding the paper disc.

The cylinder-plate method is exactly the same as the paper disc method except that the antibiotic solution is contained in open-ended cylinders placed upright on the agar media. The solution will gradually diffuse out and produce the inhibition zones.

The turbidimetric method is based on the ability of the microorganism to grow in a specified culture broth in the presence of sequenced or ordered concentrations of an antibiotic. Both the standards and the samples are handled in the same manner, and turbidity is read with a suitable turbidimeter. The turbidity increases with a decrease of antibiotic concentration or with decreased effectiveness of the antibiotic.

It is quite obvious that the first deficiency of the microbiological methods is their inability to provide information on the presence or absence of impurities, which may be microbiologically active or inactive or even toxic. This fact is especially important when the preparation contains a large proportion of impurity. The second is their inability to differentiate between the potency or effectiveness of the antimicrobial agent and its degradation products or impurities, particularly if the diffusibility of the latter is significantly greater than that of the former. It is very possible to lead to erroneous or conflicting

results (5). It should be kept in mind that the inhibitory effect is additive for the individual components, and thus a previous separation is required. In addition, microbiological methods require long (e.g. eighteen hours) periods of incubation, and the results are of low precision (±10-15% range) unless many conditions are well controlled and a large number of observations are made (6).

From the foregoing criticisms, it is apparent that there is a need for much greater specificity that only chemical or physical methods can provide. When suitable chemical or physical methods are available they are usually more reliable, reproducible, accurate, precise and furthermore less time consuming.

A simple procedure for the assay of chlortetracycline was described by Levine et al.(7), based on its conversion to anhydrochlortetracycline by heating with acid, followed by spectrophotometric determination at 440 nm. Grove and Randall in 1955 (8) described three spectrophotometric or colorimetric methods which were then in current use for the assays of tetracyclines. One (for tetracycline and chlortetracycline) was the method of Levine et al.(7), another (for tetracycline, oxytetracycline and chlortetracycline) was adapted from the work of Monastero et al.(9) involving color development of the phenolic hydroxyl group in tetracyclines with ferric chloride followed by absorbance measurement at 490 nm, and the third (for tetracycline and oxytetra-

cycline) involved the direct spectrophotometric measurement of an alkaline solution at 380 nm. A more specific way of analyzing tetracycline and chlortetracycline in the presence of each other and of oxytetracycline itself was described by Chiccarelli et al.(10). The method involves the conversion of chlortetracycline to iso-chlortetracycline by heating at pH 7.5. Tetracycline is stable under these conditions while iso-chlortetracycline is stable in hot acid. For oxytetracycline, a conversion to colorless apooxytetracycline occurs in acidic conditions. Cruceanu et al. (11) analyzed tetracycline pharmaceutical preparations simply by measuring the absorbance at 355 nm in dilute acid solution. Good agreement with microbiological results was claimed. Chatten and Krause (12) have described the quantitative determinations of various tetracyclines in pharmaceutical formulations, based on the color development resulting from the complex formed between the tetracyclines and thorium. Janik and Holiat (13) recently have described a spectrophotometric method for oxytetracycline, based on the complex formation with cerium. However, most of the colorimetric or spectrophotometric methods devised so far lack the ability to differentiate between the tetracyclines being assayed and closely related impurities or their degradation products. An attempt has been made by McCormick et al.(14) using a spectrophotometric method for the assay of epitetracycline in tetracycline, based on their small

absorbance differences in 0.1 N sulfuric acid solution at 254 and 267 nm. However, no applications to the analysis of pharmaceutical preparations have been reported, probably because of the quite small absorbance differences and the low percentage of the epi-form in the tetracycline preparations.

Numerous very sensitive fluorometric methods have been developed utilizing the pronounced fluorescence of tetracyclines especially when chelated with cations. Levine et al. (7) described a method for chlortetracycline based on the development of stable fluorescence in heated aqueous solutions buffered at pH 7.5, presumably because of conversion to iso-chlortetracycline. Kohn (15) found that tetracyclines form mixed fluorescent complexes with calcium and barbiturates which can be extracted with organic solvents such as ethyl acetate. These procedures, however, are just suitable for analysis of tetracycline, chlortetracycline, demethylchlortetracycline and rolitetracycline and are applicable even in biological samples but are not suitable for oxytetracycline because of the reduced fluorescence and extraction problems with that drug. Ibsen et al.(16) devised a method for the determination of oxytetracycline, in which oxytetracycline is extracted from acidic aqueous solutions with amyl alcohol, then extracted back into basic solution. An aqueous magnesium chloride solution as fluorescent reagent is added and the fluorescence measured

after a short time interval. The concentration of oxytetracycline is proportional to the fluorescence difference between the Mg-oxytetracycline chelate and the free oxytetracycline which is liberated by adding EDTA to the solution. Wilson et al.(17) have modified Kohn's (15) methods by using calcium barbitone as the reagent and chloroform as the extracting solvent and applied the procedure successfully to the analysis of tetracyclines in serum. Lever (18) has further combined the use of magnesium as a chelating agent with that of barbiturates in developing an extremely sensitive clinical procedure to determine tetracyclines in serum, sputum, pus, etc. Interestingly, Hawkins (19) has suggested the use of a clean-up procedure involving adsorption of tetracycline on a small "Florosil" column to determine tetracycline or demethylchlortetracycline in bone. Alykova (20) has claimed many advantages for beryllium over magnesium as a complexing cation for determining tetracycline in serum.

Several paper chromatographic procedures have been tried also. Selzer and Wright (21) employed buffered paper and mixed solvent systems for the determination of tetracycline and epitetracycline. Kelly and Buyske (22) made use of the sequestering agent, EDTA, to chelate some trace metals in the paper beforehand to improve the resolution. Addison and Clarke (23) determined the epitetracycline content of tetracycline preparations using Whatman's modified cellulose phosphate cation-exchange paper. Sina

et al.(24) described the determination of oxytetracycline in a number of pharmaceutical preparations in the presence of its degradation products.

The many advantages of thin-layer chromatography over paper chromatography have led to the almost complete replacement of the latter by the former. Kapadia and Rao (25) applying the idea of Kelly and Buyske (22), have successfully resolved tetracycline, oxytetracycline and chlortetracycline. Sonanini and Anker (26) claimed more consistent resolution of the tetracyclines by dipping the TLC plates in glycerol prior to their use. Ascione et al.(27) combined the advantages of using EDTA and a mixture of polyethylene glycol and glycerol to get better resolution of tetracycline, chlortetracycline and demethylchlortetracycline from each other and from their respective degradation products. Keiner et al.(28) and Fernandez et al.(29) have described another mobile solvent and simple layer system, which is suitable for quantitative analysis for tetracycline, epitetracycline, anhydrotetracycline and epianhydrotetracycline. Recently, van Hoeck et al.(30) have suggested another combination of stationary and developing phases as having superior resolution for tetracycline and its degradation derivatives. In a series of papers, Simmons et al.(31-33) have described systems capable of resolving tetracycline from its impurities, based on layers of microcrystalline cellulose buffered with disodium EDTA and ammonium chloride.

With larger quantities, column chromatography has been recommended. Kelly (34) has developed a method for the analysis of anhydrotetracycline and epianhydrotetracycline in tetracycline using partition chromatography. Ascione et al.(35, 36) and Fike et al.(37) have applied the information of the thin-layer chromatography system devised before (27) to analyze certain tetracyclines in the presence of their impurities. For the determination of the purity of oxytetracycline, Bailey (6) has developed another quantitative chromatographic procedures. Recently, Ascione et al.(38) proposed an efficient automated procedure of analysis for various crystalline tetracyclines or their dosage forms. In addition to the chromatographic procedures, electrophoresis is a valuable method of separation. Paris et al.(39) and Ochab (40) have investigated an analytical method using the paper electrophoresis technique for antibiotics including the tetracyclines. It should be remembered, however, that all of the chromatographic procedures mentioned above, as well as electrophoresis, are just preliminary steps to the actual quantitative determination.

The progress in high speed liquid chromatography has demonstrated it to be ideally suited to the specific quantitative and qualitative analysis of tetracyclines. Versatility, speed, excellent resolution and high sensitivity have been claimed by Butterfield et al.(41). Tsuji et al. (42) applied high pressure liquid chromatography to

determine tetracyclines and claimed a relative standard deviation of 1.02% and approximate sensitivity to 10 nanograms of tetracycline per sample injected (1.5 ul).

Visual or potentiometric non-aqueous titrations can be readily performed on both the free bases and hydrohalide salts of most of the tetracyclines. Sideri and Osol (43) have described the titrations of several tetracyclines in glacial acetic acid. Yokoyama and Chatten (44) were able to obtain quite satisfactory results using perchloric acid in dioxane as titrant, methylene blue and quinaldine red as mixed indicator, and nitromethane-formic acid-benzene system as solvent. However, for some of the pharmaceutical preparations, problems were encountered due to formulation excipients.

Aside from the tremendous value of NMR in elucidating the structure of compounds, Von Wittenau and Blackwood (45) recorded NMR (^1H) spectra of several tetracyclines using pyridine, dimethylsulphoxide and trifluoroacetic acid as solvents. The different chemical shifts for the C-4 proton in tetracycline, anhydrotetracycline, epitetracycline and epianhydrotetracycline might imply some analytical possibilities. Recently, Schlecht and Frank (46), using NMR to study tetracycline epimerization, also revealed some potential utility for this technique both in quantitative and qualitative determinations of the tetracyclines.

Optical rotatory dispersion and circular dichroism

spectra have been extended to the studies of tetracyclines. Mitscher et al.(47-50) have had a series of publications in this field.

III. Polarography

Polarography, the study of current-voltage curves obtained with mercury electrodes with periodically-renewed surfaces, was first devised by Professor J. Heyrovsky as a physicochemical method. The study of polarographic electrolysis from this point of view has contributed considerably to our understanding of transport processes, of the role of potential control in electrolysis, and of effects of chemical reactions antecedent, parallel or consecutive to the electrode process proper. Under appropriate conditions, when a definite potential is applied between the two electrodes in the polarographic cell, a typical polarogram is thus produced as shown in Figure 1.

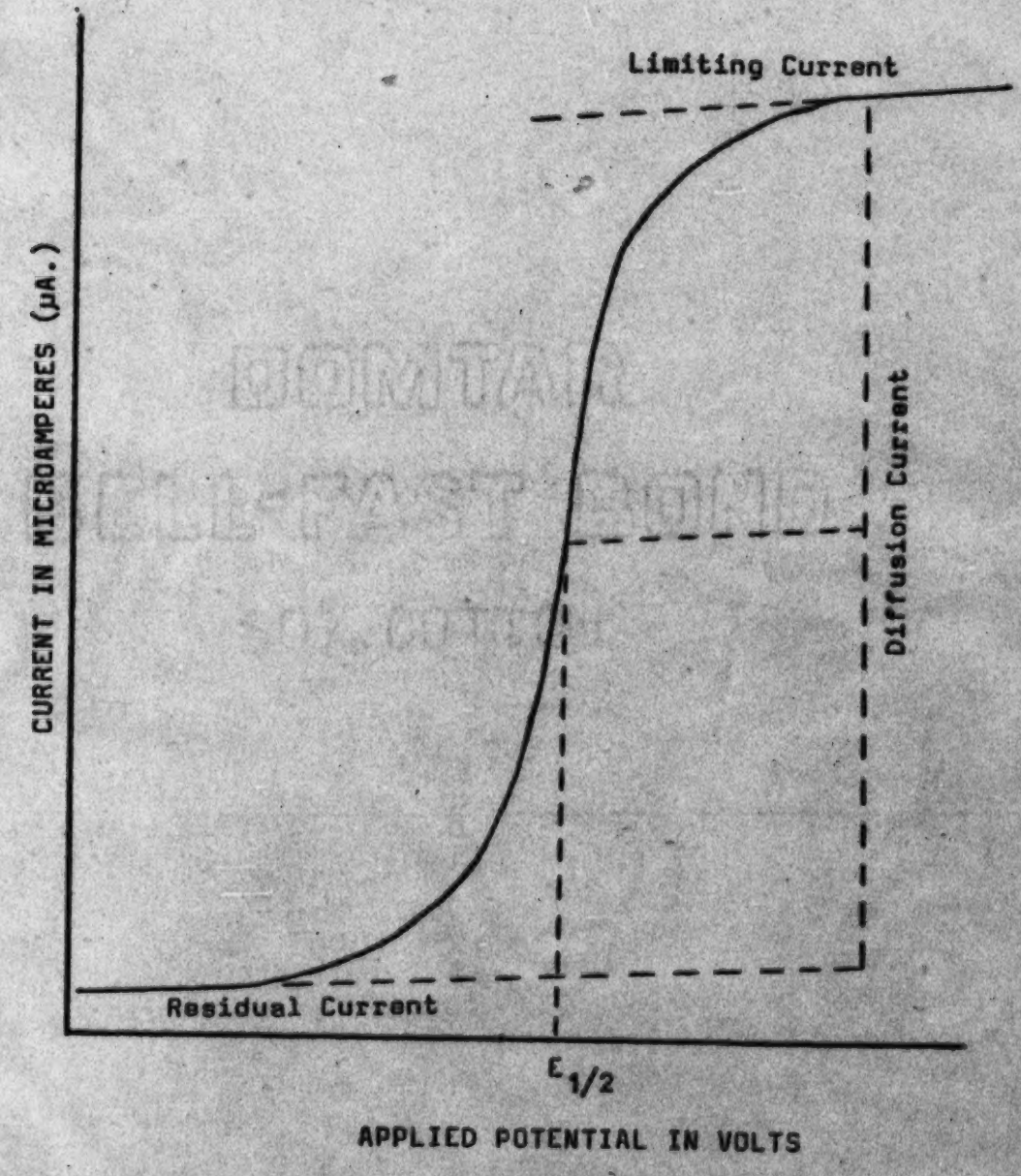
The position of the half wave potential ($E_{1/2}$ value) should be a characteristic value for a particular substance when the conditions are specified. This is the basis for qualitative identification of depolarizers.

Applying the idea of Fick's first and second diffusion laws and examining the various factors governing the diffusion current, Ilkovic (51) has derived the following equation:

$i_d = knD^{1/2}C\ m^{2/3}\ t^{1/6}$ .....(Equation 1), where

i_d = average diffusion current in microamperes (μA) during

FIGURE 1: THE NORMAL CURRENT-VOLTAGE WAVE



the life of a mercury drop.

k = a constant with a value of 607 when average diffusion current is measured.

n = the number of electrons per ion or molecule of the electroactive species involved in the electrode reaction.

D = diffusion coefficient of reducible or oxidizable substance in cm^2/sec .

C = concentration of electroactive species in millimoles per liter.

m = rate of mercury flow from dropping mercury electrode (DME) capillary in mgs/sec .

t = mercury dropping time in seconds, usually counted at the potential of measuring diffusion current.

Based on the direct proportionality relationship between the diffusion current and concentration, polarography thus has been applied widely as a quantitative analytical tool. The sensitivity, the small volume of sample, and in particular the selectivity of polarographic methods are the main factors leading to their present situation.

The theoretical principles of polarography are beyond the scope of the present studies but have been discussed in detail in a number of textbooks (52, 53).

It should be noted that, in the Ilkovic equation, the rate of mercury flow, m, and dropping time, t, are

greatly influenced by the mercury column height, h. But actually the net pressure at the DME capillary tip is equal to the observed height of mercury column minus the back pressure, which is due to the excess pressure needed to overcome the interfacial tension of mercury and to expand the drop. The general equation can be summarized as follows:

Corrected height of mercury column in cm = observed height of mercury column in cm - back pressure

or, $h_{corrected} = h_{observed} - \frac{3.1}{m^{1/3} t^{1/3}}$ (when in aqueous solution) .....(Equation 2)

From the fact that an increase in the corrected height of the mercury column produces no increase in mercury drop size, but rather increases the number of drops formed per unit of time, the relationship between m and the corrected height of the mercury column can be shown as:

$m = k_1 h_{corrected}$ .....(Equation 3), where k_1 is a constant.

Meanwhile, the value of dropping time, t , varies inversely with the corrected height of the mercury column, as shown by Equation 4:

$t = \frac{k_2}{h_{corrected}}$ .....(Equation 4), where k_2 is another constant.

If Equation 3 and 4 are substituted into the Ilkovic equation, Equation 1, and providing all other factors which can affect diffusion current remain constant, then another

simplified relationship between diffusion current and corrected height of mercury column can be established as: $i_d = k_3\sqrt{h_{corrected}}$ .....(Equation 5), where k_3 is also a constant.

Equation 5 provides a means of ascertaining whether the current produced at the DME is actually the result of a diffusion controlled process.

The dropping time, t , also varies as a function of applied voltage, first increasing with the applied potential up to -0.52 V (vs. saturated calomel electrode) and then decreasing sharply. But, usually it is controlled within the order of 2 to 5 seconds.

Although the temperature term does not appear in the Ilkovic equation, a temperature change can affect every term except n. The diffusion coefficient, D , is especially sensitive to the change of temperature which can increase the diffusion current by 1-2% per degree rise in temperature in the vicinity of 25.0° C . Therefore, careful control of the temperature of the electrolysis cell is essential.

Complex formation of the electroactive species with any component of the supporting electrolyte or other impurities may also profoundly affect the diffusion coefficient.

The occurence of maxima is undesirable because of the interference with diffusion current measurement. The addition of an appropriate amount of a suitable maxima suppressor (usually the final concentration of maxima suppressor

in solution ranges between 0.001-0.01%) is thus suggested. A high concentration of maxima suppressor is to be avoided because it may markedly change the viscosity of the solution and even markedly suppress the normal waves.

Due to the oxygen waves appearing at about -0.10 V and -0.90 V (vs. SCE), the procedure of deaeration is necessary. The air is usually removed by bubbling some inert gas such as oxygen-free nitrogen through the solution for about 10 minutes prior to analysis (depending on the volume of solution and the geometry of the polarographic cell) and allowing a gentle flow of nitrogen to layer above the solution surface to prevent oxygen reabsorption during analysis. A purified grade of commercial tank nitrogen is frequently adequate for this purpose. If the last trace of oxygen must be removed, the nitrogen should either be passed through a heated tube of copper turnings or a gas washer containing some suitable reducing agent (54, 55).

There are several ways of evaluating the unknown concentration of a substance by polarographic analysis among which the following are given:

(i) Absolute Method:

The absolute method is based on the direct application of the Ilkovic equation, however, for obvious reasons, it is not used to any extent in practical work.

(ii) A Modified Absolute Method:

The Ilkovic equation can be rearranged to

$$knD^{1/2} = \frac{i_d}{C\, m^{2/3}\, t^{1/6}}$$

The characteristics which are independent of the electrodes and instrument are grouped together on the left and are collectively referred to as the "diffusion current constant", I_d, which is frequently available in the literature with reproducibility within 5% under specified conditions. Then rearrangement of the above equation gives,

$$C = \frac{i_d}{(knD^{1/2})\, m^{2/3}\, t^{1/6}} = \frac{i_d}{I_d\, m^{2/3}\, t^{1/6}} .$$

Although the concentration is obtained in this manner, the disadvantages of this method are that it is very time consuming and not very accurate.

(iii) Direct Comparison by Calibration Curve Method:

This method is convenient for routine analyses of large numbers of samples. However, calibration curves should not be assumed to be valid from day to day, but have to be verified for each series of analyses.

(iv) Alternate Direct Comparison Method:

This method is based on the simple relationship

$$\frac{(i_d)_{\text{sample}}}{(i_d)_{\text{standard}}} = \frac{C_{\text{sample}}}{C_{\text{standard}}}$$

Greatest accuracy is obtained when the concentrations of standard and sample closely approximate one another, especially for a slightly non-linear relationship between wave height and concentration.

(v) Standard Addition Method:

In this method the diffusion current of the unknown solution with volume A is determined first and then a known volume B of standard solution is added to the unknown solution and finally the diffusion current of the mixed solution is measured. The unknown concentration is then calculated from the following equation:

$$C_x = C_{standard} \cdot \frac{B\, i_{d1}}{i_{d2}\,(A+B) - A\, i_{d1}}$$, where

C_x = concentration of the unknown test solution.

$C_{standard}$ = concentration of the standard solution.

A = volume of the test solution.

B = volume of the standard solution added.

i_{d1} = diffusion current of the unknown test solution.

i_{d2} = diffusion current of the mixed solution.

Usually the maximum accuracy is obtained when the wave height caused by the addition of the standard solution is approximately double that of the unknown test solution alone. If everything is handled properly, this method is claimed to be more accurate than the calibration curve method, but more time consuming.

The polarographic method of analysis is very suitable for the analysis of inorganic matter since so many elements and inorganic compounds are reduced at the dropping mercury electrode, giving well defined waves. Accordingly, this method has found wide application in the field of

metallurgical analysis for determining the component parts or impurities of alloys (56). The determination of trace metallic impurities in commercial chemicals, or trace inorganic constituents in water, in petrol, in various organic and biological materials and in foodstuffs also have been reported. A large number of examples have been collected by Purdy (57).

A great variety of organic compounds, including many ketones, aldehydes, alkenes, aryl alkynes, azomethines, nitriles, azo compounds, azoxy compounds, peroxy compounds, disulfides, nitro compounds, nitroso compounds, hydroxylamines, nitrites, nitrates, quinones, aryl halides, polynuclear aromatic ring systems, heterocyclic double bonds, etc. are reducible at the dropping mercury electrode. Zuman (58, 59) has collected and classified these various papers.

Only over the past decade has it been recognized that polarography can be useful in solving some fundamental problems in chemistry. Especially in organic chemistry, polarography can be used in the determination of equilibrium and rate constants, in studies of reaction mechanisms, in the search for optimum conditions for some preparative reactions, in studies and comparisons of reactivities of organic compounds, and in correlations of structure with polarographic data (58, 59).

Polarographic analysis is not limited to aqueous

solutions, and can be performed in some non-aqueous systems (60-62). Usually the organic solvents are sufficiently polar to dissolve some organic compounds which may not be soluble in water.

Of practical interest to this thesis, polarographic techniques have been applied on a limited basis to studies of tetracycline antibiotics. In 1952, Doskocil and Vondracek (63) determined chlortetracycline. Two years later Doskocil (64) extended the work to mixtures of oxytetracycline and chlortetracycline. Doskocilova (65) has reported on the polarographic behaviour of various tetracyclines and their degradation products. In 1963, Doan and Riedel (66) proposed the use of polarographic measurements for quantitative and qualitative purposes for "pure" tetracycline hydrochloride. Hetman (60) at about the same time, used differential cathode ray polarography to record some tetracyclines, and introduced the use of non-aqueous solvent systems. Alternating-current polarography has been applied to the assays of pharmaceutical preparations of different tetracyclines by Caplis et al.(67). Later in Caplis' Ph. D. thesis (62), a detailed study of the electroreduction mechanism was given. Recently, Silvestri (68) has described the AC and DC polarographic determinations of several pharmaceuticals (including doxycycline). His method was reported to be suitable for automated determinations, and was applied to the determination of content uniformity.

STATEMENT OF THE PROBLEM

Polarography is a rapid, precise and accurate method for both quantitative and qualitative analytical purposes. Some research workers have determined certain tetracyclines polarographically. However, there are deficiencies and discrepancies in the literature and a systematic study appeared to be of value. In the present work, efforts have been made to investigate the development of a quantitative dc polarographic method of analysis for certain tetracyclines and their pharmaceutical dosage forms, and some preliminary studies regarding their determination in some biological samples.

In order to accomplish the foregoing, the following parameters have been systematically investigated:

(i) The determination of the optimum buffer system (i.e. that which offers the least interference to the polarographic waves of the tetracyclines.

(ii) The determination of the optimum pH within the chosen buffer system which would give the best resolved waves and afford the greatest stability.

(iii) Studies of the effect of pH on $E_{1/2}$.

(iv) Studies of the effects of mercury column height on mercury flow rate, m, dropping time, t, and the diffusion current. The determination of diffusion dependency by plotting diffusion current versus (corrected mercury column height)$^{1/2}$.

(v) The preparation of a calibration curve for the various

tetracyclines by plotting the total current versus concentration, at the optimum conditions determined earlier.

(vi) The application of the method to the analysis of pharmaceutical dosage forms and a comparison of the results with those provided by the manufacturers.

(vii) The investigation of some extraction procedures for separation of the tetracyclines from biological samples and then a determination of the tetracyclines polarographically.

(viii) The examination of certain fundamentals pertaining to the process of electroreduction.

EXPERIMENTAL

Apparatus

Standard laboratory glassware; Sargent model XV recording polarograph; polarographic cell (30 ml) with saturated calomel electrode as reference; one meter ruler; stop watch; purified tank nitrogen; thermostatically controlled water bath with a pump system; Corning pH meter fitted with glass-calomel electrode system; Mettler gramatic balance; hot plate; water bath; titration lamp; magnetic stirring apparatus; vacuum device.

Reagents and Solutions

The water should be at least double-distilled and polarographically pure. All chemicals employed in this study were either A.C.S. or reagent grade in quality.

Glacial acetic acid; 0.05 N perchloric acid in dioxane standardized against primary standard potassium acid phthalate; 0.5% crystal violet in glacial acetic acid; 6% mercuric acetate in glacial acetic acid; 0.2 M boric acid stock solution; 0.05 M borax stock solution; 1% freshly prepared gelatin solution; cadmium chloride; hydrochloric acid; disodium EDTA.

Reference Standards

The following is a list of pure drugs employed as reference standards in this study.

Drug	Manufacturer
Tetracycline Hydrochloride	Cyanamid of Canada Limited
Oxytetracycline Hydrochloride	Pfizer Company Limited

Chlortetracycline Hydrochloride	Cyanamid of Canada Limited
Demethylchlortetracycline Hydrochloride	Cyanamid of Canada Limited
Tetracycline Phosphate Complex	Bristol Laboratories of Canada Limited

Procedure

A. Determination of Reference Standard Purity

The non-aqueous titration system devised by Sideri and Osol (43) was employed to determine the purity or potency of reference standards. A quantity of about 200 mg of the crystalline salt was accurately weighed and dissolved in 80 ml of glacial acetic acid. In order to create a readily titratable species, 10 ml of 6% mercuric acetate in glacial acetic acid was added to the solution and 2 drops of 0.5% crystal violet in glacial acetic acid was used as the indicator. With the aid of a magnetic stirring apparatus and a titration lamp, the solution was first stirred for 5 minutes and then titrated to a blue green endpoint with 0.05 N perchloric acid in dioxane using a 10 ml burette. The progress of the titration was also followed potentiometrically with a Corning pH meter equipped with a glass-calomel electrode combination. In the above titrations, it was very important to protect the system from atmospheric moisture.

B. Constant Temperature Control for Polarography

The polarographic cell was immersed in a water bath to about 5/6 of its height. The water bath was connected to a large thermostatically controlled reservoir and the water was circulated by means of a pump. The temperature of the circulating water was thus kept constant at 25±0.2°C.

C. Determination of Optimum Height of Mercury Column

A standard $CdCl_2$ solution of 1.0×10^{-4} M, acidified with HCl, was prepared and a 25 ml aliquot was transferred to the polarographic cell. After deaeration for 10 minutes with purified nitrogen gas, the solution was protected from oxygen reabsorption by applying a gentle flow of nitrogen gas over the solution surface. The polarograms were run separately at various heights of the mercury column ranging from 50.0 cm to about 90.0 cm with 5 cm as the interval. The dropping time in each instance was recorded, the polarograms were compared and the optimum height was thus determined.

D. Determination of Optimum Buffer System and Optimum pH

A variety of buffer systems such as the Britton-Robinson buffer (universal buffer system covering the pH range of 2.6 to 11.8), glycine-HCl buffer (pH 2.2 to 3.6), lithium acetate-acetic acid buffer (pH 3.6 to 5.6), phthalate-NaOH buffer (pH 4.2 to 6.0), barbital buffer (pH 6.8 to 9.2), phosphate buffer (pH 5.7 to 8.0), boric acid-borax

buffer (pH 7.6 to 9.2) and borax-NaOH buffer (pH 9.3 to 10.0), with 0.2 pH unit as the interval were prepared. Each of the above buffer solutions was scanned polarographically. Then the concentration of each tetracycline hydrochloride in the individual buffer solution was maintained at about 1.0×10^{-4} M. After deaeration of the solution, the polarograms were successively obtained and then compared. From these, the most acceptable ones were selected. The optimum pH within the chosen buffer system was determined examining the effect of smaller pH changes (eg. 0.1 pH unit) as the interval.

E. Determination of Solution Stability

To determine the period of stability, solutions of each tetracycline salt were prepared at a concentration of about 1.0×10^{-4} M using the optimum buffer solutions determined earlier. The solutions were protected from light as much as possible and the polarograms were determined every half hour from the time of preparation. The length of time during which no significant change occurred in the polarogram was determined for each of the tetracyclines.

F. Determination of pH Change Before and After Electrolysis

The pH values of the solutions prior to and after running the polarograms were measured to insure that the pH had remained constant.

G. Determination of Diffusion Dependency

All solutions were prepared at about 1.0×10^{-4} M using their specified pH buffer solutions. By arbitrarily setting the height of the mercury column ranging from 56.0 cm to about 90.0 cm, the polarograms were successively determined. Meanwhile, the dropping time, t , and mercury flow rate, m , were recorded at a specified potential (tetracycline at -1.70 V vs SCE; oxytetracycline at -1.60 V vs SCE; chlortetracycline at -1.66 V vs SCE and demethylchlortetracycline at -1.70 V vs SCE) for each height setting. For demethylchlortetracycline, the addition of 0.125 ml (from calibrated dropper) of 1% freshly prepared gelatin solution to the 25 ml of solution was necessary.

H. Preparation of Calibration Curves

All calibration curves were made by averaging five determinations and all current readings were corrected to account for the degree of purity or potency of the reference standards being used.

(i) Calibration Curve for Tetracycline Hydrochloride:

A quantity of the hydrochloride equivalent to about 50 mg was accurately weighed. This amount of crystalline material was dissolved, quantitatively transferred and made to volume with the optimum pH buffer solution previously determined (pH 7.75 boric acid-borax buffer) in a 100 ml volumetric flask. This constituted the stock solution having a concentration of 0.5 mg tetracycline·HCl/ml .

Dilutions were then made from the stock solution with buffer solution to contain 0.100 mg, 0.250 mg, 0.375 mg, 0.500 mg, 0.750 mg, 1.000 mg and 1.250 mg per 10 ml solution respectively. A 25.0 ml aliquot of the standard solution was pipetted into the polarographic cell already equilibrated at 25.0° C and then deaerated by bubbling the purified tank nitrogen through the solution for 10 minutes. A gentle flow of the nitrogen gas was applied to the layer above the solution surface during the analysis. The applied potential range was 0.00 to -2.00 V and the sensitivity scale was set at 0.015 uA/mm. The polarograms were determined and the total currents were measured at the potential which is generally the center point of the plateau (i.e. -1.70 V vs SCE in this case).

(ii) Calibration Curve for Oxytetracycline Hydrochloride:

The procedure was identical to that for tetracycline hydrochloride except that the optimum pH of the boric acid-borax buffer was chosen as 8.20 and the total currents were measured at -1.60 V vs SCE.

(iii) Calibration Curve for Chlortetracycline Hydrochlorides:

The procedure was identical to that for tetracycline hydrochloride except that the optimum pH of the boric acid-borax buffer was selected as 7.95 and the total currents were measured at -1.66 V vs SCE.

(iv) Calibration Curve for Demethylchlortetracycline Hydrochloride:

The procedure was identical to that for tetracycline hydrochloride. However, the addition of an appropriate amount of freshly prepared 1% gelatin solution was required for this drug (0.125 ml of gelatin solution added to the 25 ml solution with a calibrated dropper) during the deaeration step.

I. Quantitative Assay of Pharmaceutical Dosage Forms

General Procedure # 1 --- A suitable quantity of the powdered material was accurately weighed into a 150 ml beaker, 90 ml of the specified buffer solution was added and the mixture stirred for 10 minutes. Upon the completion of the stirring, the mixture was filtered under suction through a sintered glass filter with medium porosity, after which tha clear filtrate was quantitatively transferred to a 100 ml volumetric flask and brought to volume with the specified buffer solution. The resulting stock solution had a concentration of approximately 5.0 mg of the tetracycline base or salt per 10 ml of solution. A fifteen ml. aliquot of the stock solution was pipetted into each of five 100 ml volumetric flasks, and made up to volume with the buffer solution. The pH of the solution was checked and adjusted before the electrolysis. The deaeration step was carried out as described previously and the polarogram was determined. When the other compounds in the preparations were declared on the label, solutions of those compounds were prepared at the appropriate concentration, and

their polarograms determined so that the currents attributable to those compounds could be calculated.

General Procedure # 2 --- The liquid preparation was vigorously shaken for at least 10 minutes and then a suitable quantity of it was accurately pipetted into a volumetric flask and finally diluted to volume with the optimum buffer solution. This was followed by magnetic stirring for 5 minutes so that the stock solution prepared had a theoretical concentration of 5.0 mg of the tetracycline base or salt per 10 ml of solution. If the tetracyclines existed as insoluble forms, such as free base or some complex, the addition of an appropriate amount of 3 M hydrochloric acid was necessary immediately after pipetting the original sample. The remainder of the method was identical to General Procedure # 1, starting with the words "A fifteen ml aliquot of the stock solution was pipetted into each of five 100 ml volumetric flasks -----".

The following is a list of the commercial products that were assayed in this investigation:

(i) Tablets:

Trade Name	Drug	Manufacturer
Declomycin 300	DMCTC·HCl	Cyanamid of Canada Ltd.
T-Tabs	TC·HCl	ICN Canada Ltd.
Novotetra	TC·HCl	Novopharm Ltd.

For each product, ten tablets were weighed and the

mean weight was determined. The ten tablets were ground to a fine powder using a mortar and pestle, then General Procedure # 1 was followed directly.

(ii) Capsules:

Trade Name	Drug	Manufacturer
Tetrex 100 mg	TC·Phosphate	Bristol Lab. of Canada
Tetrex 250 mg	TC·Phosphate	Bristol Lab. of Canada
Tetrex bid CAPS 500 mg	TC·Phosphate	Bristol Lab. of Canada
Tetrex-F	TC·Phosphate & Nystatin	Bristol Lab. of Canada
Achromycin	TC·HCl	Cyanamid of Canada Ltd.
Aureomycin	CTC·HCl	Cyanamid of Canada Ltd.
Declomycin	DMCTC·HCl	Cyanamid of Canada Ltd.
T-Caps	TC·HCl	ICN Canada Ltd.
Tetralean	TC·HCl	M.T.C. Pharmaceuticals Ltd.
Novotetra	TC·HCl	Novopharm Ltd.
Tetracyn	TC·HCl	Pfizer Company Ltd.
Terramycin	OTC·HCl	Pfizer Company Ltd.

For each product ten capsules were weighed and then their contents were transferred to a clean beaker, the last traces of the contents being removed by a vacuum device. Following this, the tare weight of each capsule was determined and the net weight of the content was thus found.

General Procedure #1 was then followed in each instance.

(iii) Injection Powders in Vials:

Trade Name	Drug	Manufacturer
Achromycin for IM	TC·HCl	Cyanamid of Canada Ltd.
Tetracyn for IM	TC·HCl	Pfizer Company Ltd.
Tetracyn for IV	TC·HCl	Pfizer Company Ltd.
Terramycin for IM	OTC·HCl	Pfizer Company Ltd.
Terramycin for IV	OTC·HCl	Pfizer Company Ltd.

The procedures for obtaining the mean weight of the contents were similar to those for capsules. Having removed the powder from the vials, General Procedure #1 was followed directly.

(iv) Ointments:

Trade Name	Drug	Manufacturer
Achromycin Eye-Ear Ointment	TC·HCl	Cyanamid of Canada Ltd.
Achromycin Topical Ointment	TC·HCl	Cyanamid of Canada Ltd.
Aureomycin Ophthalmic Ointment	CTC·HCl	Cyanamid of Canada Ltd.
Aureomycin Topical Ointment	CTC·HCl	Cyanamid of Canada Ltd.
Terramycin Ophthalmic Ointment	OTC·HCl & Polymyxin B Sulfate	Pfizer Company Ltd.
Terramycin Topical Ointment	OTC·HCl & Polymyxin B Sulfate	Pfizer Company Ltd.

The stock solution was prepared by successive heating, stirring, cooling and filtering of a suitable accurately known weight of ointment with 90 ml, 50 ml, 30 ml and 20 ml, respectively, of specified buffer solution. The accumulated grease-free filtrate was then quantitatively transferred into a 200 ml volumetric flask and made up to volume with the buffer solution. The stock solution was then diluted according to General Procedure #1 prior to obtaining the polarogram.

(v) Syrups:

Trade Name	Drug	Manufacturer
Tetrex	TC·HCl	Bristol Lab. of Canada
Achromycin	TC	Cyanamid of Canada Ltd.
Terramycin	Ca·OTC	Pfizer Company Ltd.

For the foregoing products, General Procedure #2 was followed with a minor modification. For Terramycin Syrup, the further addition of three moles of disodium EDTA per mole of calcium oxytetracycline complex was necessary when preparing the "stock" solution.

(vi) Suspensions:

Trade Name	Drug	Manufacturer
T-Liquid	TC	ICN Canada Ltd.
Novotetra	TC	Novopharm Ltd.
Tetracyn	TC	Pfizer Company Ltd.

For the above pharmaceuticals, General Procedure #2 was employed.

(vii) Pediatric Drop:

Trade Name	Drug	Manufacturer
Terramycin	Ca·di-OTC	Pfizer Company Ltd.

The sample treatment of this preparation was similar to that for Terramycin Syrup.

J. Quantitative Analysis of Tetracyclines in Blood and Urine

The extraction procedures for oxytetracycline hydrochloride from blood devised by Scales et al.(69) were modified and applied. To the 20 ml of urine sample, 20 ml of 0.1 N HCl containing β-mercaptopropionic acid and 5 ml of 30% w/v trichloroacetic acid were added and mixed thoroughly for 5 minutes. The mixed solution was then centrifuged at 2000 rpm for ten minutes. The supernatant liquid was removed and extracted by a total of 100 ml of ether to remove any traces of trichloroacetic acid. Then pH adjustment had to be carried out for the ether-free aqueous layer by adding 2 ml of 1.0 N NaOH and 12 ml of citrate-phosphate buffer. Under these buffered conditions, extraction was performed by addition of 200 ml of freshly distilled ethylacetate and centrifugation to get the upper layer, the ethylacetate layer. Finally, 2.0 ml of β-mercaptopropionic acid in methanol (50 mg/ml) was added to this ethylacetate

layer, the mixture was then evaporated with a flash-evaporator at a temperature of 60°C. The residue was dissolved in 25 ml of the specified pH boric acid-borax buffer and transferred to the polarographic cell for determination.

RESULTS AND DISCUSSION

A. Reference Standard Purity

The non-aqueous titration system devised by Sideri and Osol (43) was found to be suitable for the determination of the purity of the various tetracycline hydrochloride reference standards with the exception of tetracycline phosphate complex. By utilizing glacial acetic acid as an amphiprotic solvent, the weak basicity of the tetracyclines was so enhanced that a very distinct visual endpoint could be easily observed.

In addition to the visual titration values, potentiometric titrimetric results were obtained concurrently and thus accurate values of purity could be calculated for the tetracycline reference standards. Both in the visual and potentiometric titrations, it was essential to protect the titration vessel from atmospheric moisture. Failure to observe this precaution resulted in over-titration, and indeed, in some instances, no endpoint was detectable.

The results of the titrations are presented in Table I together with the average percent purity for all the reference standards except the tetracycline phosphate complex. It was not possible to determine the purity of this substance by this method because the molecular weight of the complex may vary from batch to batch. For tetracycline hydrochloride and demethylchlortetracycline hydrochloride, the potencies were found to be slightly in excess of one hundred percent. These values could be considered to be

within the experimental error of the method or they could be rationalized as being mainly due to the existence of trace amounts of lower molecular weight titratable impurities. Attempts were made to purify the other two tetracyclines by means of column chromatography but this was abandoned after several trials. It is not possible to rely on the stability of these antibiotics over the long elution period and during the drying process.

B. Temperature Control for Polarographic Experiments

Although the Ilkovic equation does not contain a temperature factor, the influence of temperature variations on polarographic measurements is well documented. It has been found that the current heights have a temperature coefficient around 2percent at the room temperature. In order to circumvent this problem, a thermostatically controlled water bath and pump system was employed. The temperature of the polarographic cell was thus maintained at $25 \pm 0.2^{\circ}C$ throughout this study. Such a small variation in temperature would not have a significant effect on the current height.

C. Determination of Optimum Height of Mercury Column

The results showed that polarograms determined between heights of 56.0 and 90.0 cm were quite acceptable, however, there was a tendency for a maximum to appear when the mercury column height was lower that 55.0 cm. When the height was raised above 90.0 cm, the dropping time was shortened so that the mercury drops created a severe "swirling effect"

Table I Analysis of Tetracycline Reference Standards by Non-aqueous Titration

Drug	Sample Wt. in mg	*ml of Titrant	% Purity	Average % Purity
TC·HCl	203.26	8.38	100.13	
(Cyanamid)	205.70	8.48	100.12	100.10
	208.30	8.58	100.04	
OTC·HCl	199.20	7.68	96.75	
(Pfizer)	201.36	7.77	96.83	96.81
	195.60	7.55	96.86	
CTC·HCl	224.76	8.45	97.84	
(Cyanamid)	211.70	7.95	97.73	97.90
	212.16	8.00	98.13	
DMCTC·HCl	199.59	7.93	100.59	
(Cyanamid)	205.10	8.15	100.60	100.61
	201.72	8.02	100.65	
TC·Phos-phate (Bristol)	-	-	-	-

1) TC is tetracycline.

2) OTC is oxytetracycline.

3) CTC is chlortetracycline.

4) DMCTC is demethylchlortetracycline.

*The titrant used was 0.0505 N PERCHLORIC ACID in DIOXANE.

in the solution. A satisfactory compromise between these two extremes led to the choice of a fixed height at 65.0 cm. This led to a reasonable and satisfactory dropping time of around three seconds.

D. Determination of Optimum Buffer System and Optimum pH

A variety of buffers were prepared at 0.2 pH unit intervals. They were (a) Britton-Robinson universal buffer (pH 2.6 to 11.8); (b) glycine-HCl buffer (pH 2.2 to 3.6); (c) lithium acetate-acetic acid buffer (pH 3.6 to 5.6); (d) potassium acid phthalate-NaOH buffer (pH 4.2 to 6.0); (e) sodium barbital-HCl buffer (pH 6.8 to 9.2); (f) phosphate buffer (pH 5.7 to 8.0); (g) boric acid-borax buffer (pH 7.6 to 9.2); and (h) borax-NaOH buffer (pH 9.3 to 10.0). Each of the foregoing buffer systems was scanned polarographically and found to contain no substance that interfered with the reduction of the tetracyclines at the chosen pH. When solutions of the various tetracycline hydrochlorides were prepared with the individual buffer solutions and polarographed, however, only a few polarograms were found to be of analytical value. Figure 1 represents an ideal current-voltage plot which illustrates a well-defined limiting current plateau. The diffusion current of such a polarogram is easily measured. Because of the complex nature of the molecules, such well-defined electrocapillary curves were not obtained with the tetracyclines in any of the buffer systems investigated.

Figure 2 illustrates the current-voltage curves for tetracycline hydrochloride at various pH values in the Britton-Robinson buffer system. In view of the fact that the above mentioned buffer system covers such a wide pH range (2.6 to 11.8), it was expected that an optimum pH would be found where the polarograms of the antibiotics would be well-defined. The fact that no such pH range was apparent with this buffer may be attributed to something in its composition. In its dual capacity as supporting electrolyte, it apparently exerted a profound effect upon the reduction mechanism of the depolarizer.

At low pH values, there may well be a re-orientation of the adsorbed molecules on the electrode which may adversely influence the polarograms. At high pH values (beginning at 9.0 and above) chemical changes could be readily detected in the system as observed by rapid color changes. Therefore, it appeared to be important to limit the pH of the solutions to the general area around the neutral region.

Figures 3 to 8 inclusive represent the polarograms obtained when tetracycline hydrochloride was reduced polarographically in certain other buffer systems. An examination of these Figures quickly illustrates that the respective waves possessed one or more highly undesirable characteristics. For example, in the glycine-HCl buffer (Figure 3) as well as lithium acetate-acetic acid buffer (Figure 4), potassium acid phthalate-NaOH buffer (Figure 5) and the borax-

NaOH buffer (Figure 8), the waves were poorly resolved and consequently of no value for quantitative purposes.

On the other hand, in both the phosphate buffer (Figure 6) and the sodium barbital-HCl buffer (Figure 7), significant and prominent maxima occurred frequently.

Of the systems investigated, the boric acid-borax buffer was found to offer the optimum conditions for several reasons. Firstly, in the applied potential range of zero to two volts, an ideally linear residual current was observed. Secondly, a smooth but distinctive polarogram with a broad limiting current plateau for the second wave was obtained. Thirdly, maximum suppressor was required in only one instance. And finally, no additional supporting electrolyte was required.

Figure 9 illustrates the polarograms obtained in boric acid-borax buffer for tetracycline hydrochloride. An examination of the various polarograms resulted in the selection of pH 7.75 as providing optimum conditions for this antibiotic. Figure 10 presents the current-voltage curves obtained for oxytetracycline hydrochloride in boric acid-borax buffer. A pH value of 8.20 was selected for this drug. The current-voltage curves for chlortetracycline hydrochloride in boric acid-borax buffer are presented in Figure 11. A pH value of 7.95 was chosen for this substance. Similarly, Figure 12 represents the polarograms for demethylchlortetracycline hydrochloride in the same buffer

system. Maxima are apparent over the entire range of this buffer. However, the addition of 0.125 ml of 1% freshly prepared gelatin solution per 25.0 ml of solution successfully eliminated the maximum at pH 7.75. Consequently, this was selected as the working pH for demethylchlortetracycline.

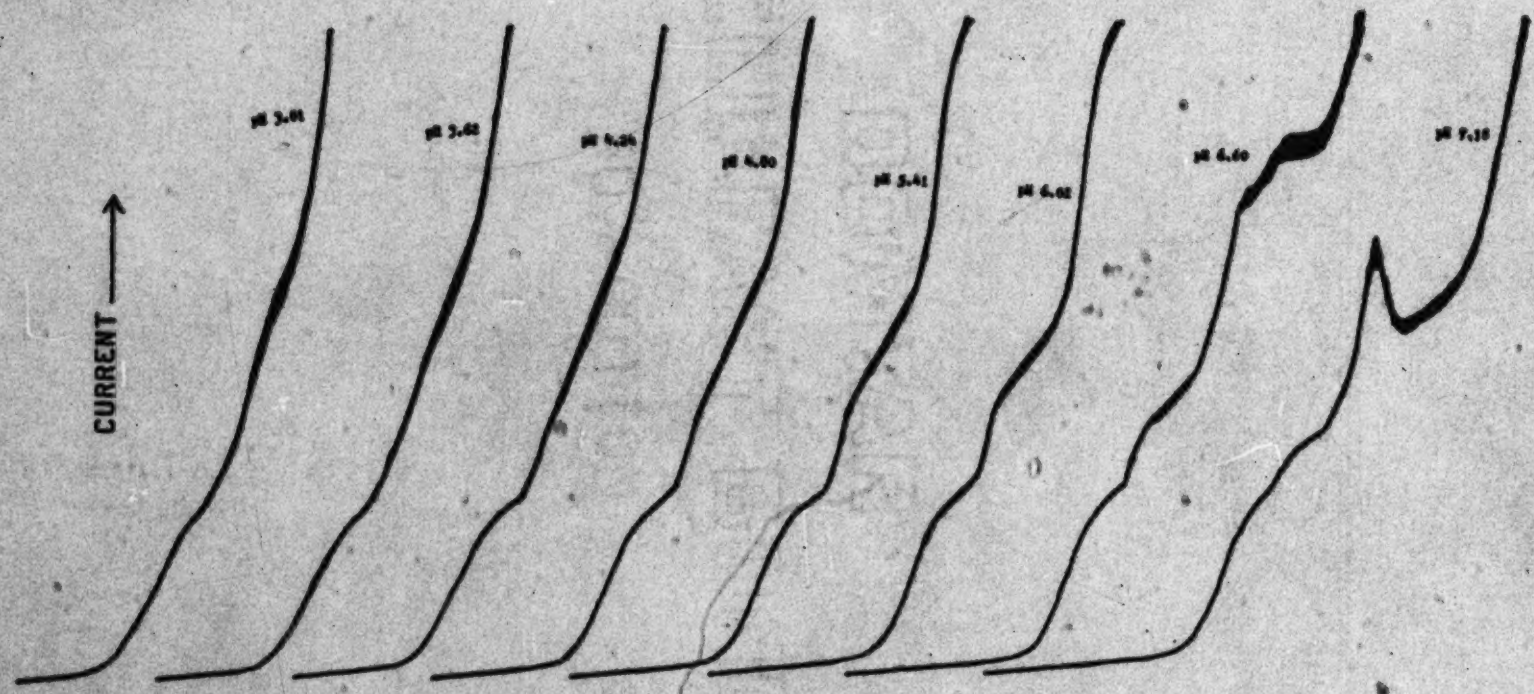
The recorded polarograms indicate there are at least two polarographic reduction steps, however, the first wave was not well resolved before the second wave made its appearance. Since neither the limiting current of the first wave nor the base line of the second wave could be identified with any degree of certainty, it was virtually impossible to measure accurately the height of either wave. This is illustrated in Figure 13. Consequently, the total current was measured for each tetracycline.

It was observed that the potential corresponding to the point at the center of the limiting current plateau was characteristic, when the total current of tetracycline hydrochloride was measured at -1.70 V vs SCE. For oxytetracycline hydrochloride, the total current was measured at -1.60 V; for chlortetracycline hydrochloride measurements were made at -1.66 V; for demethylchlortetracycline hydrochloride at -1.70 V. In all instances, a SCE was employed.

E. Determination of Solution Stability

The solutions of each of the tetracycline salts were prepared at a concentration of about 1.0×10^{-4} M using the optimum buffer system previously described. Polarograms

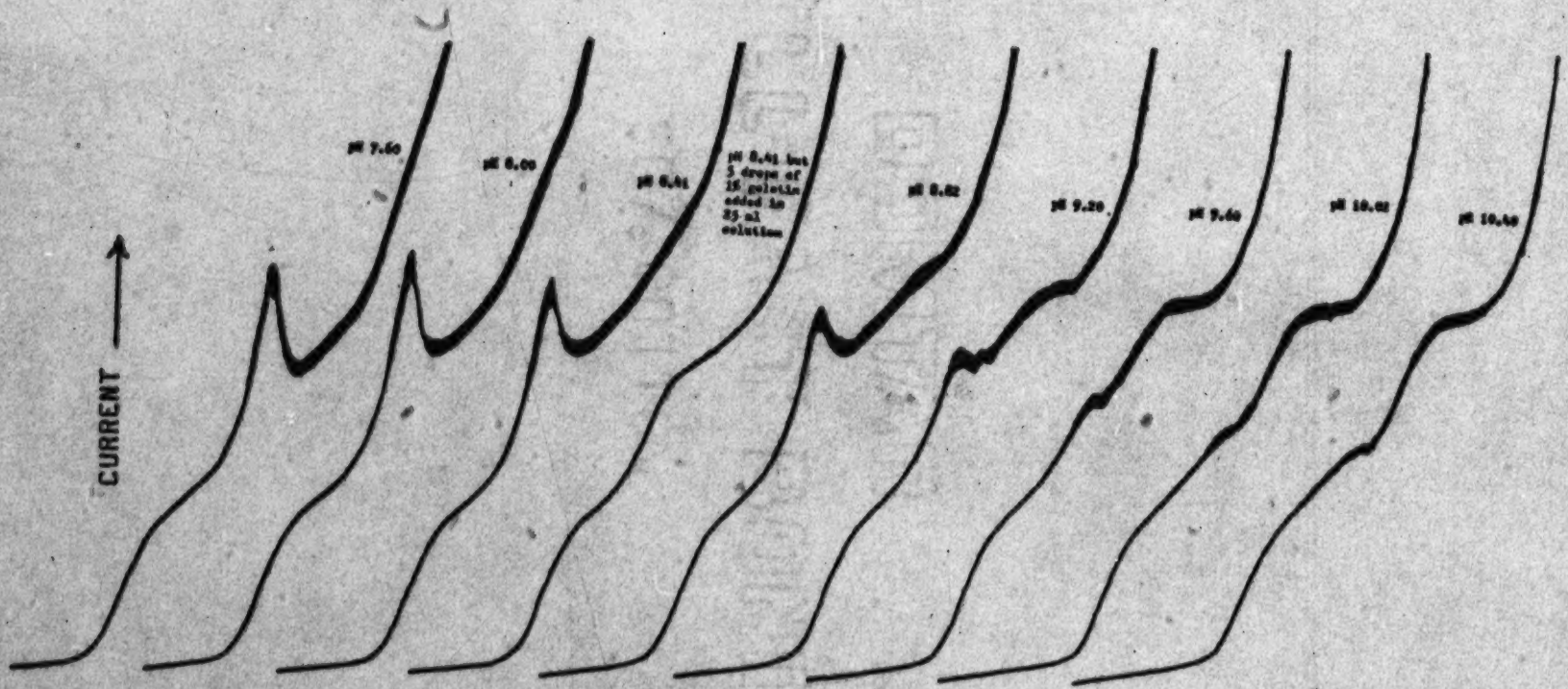
FIGURE 2: CURRENT-VOLTAGE CURVES OF TETRACYCLINE HYDROCHLORIDE IN BRITTON-ROBINSON BUFFERS



Each segment begins at -0.60 V. Each additional 0.10 V is represented by ⊢⊣

Concentration = 1.11×10^{-4} M.

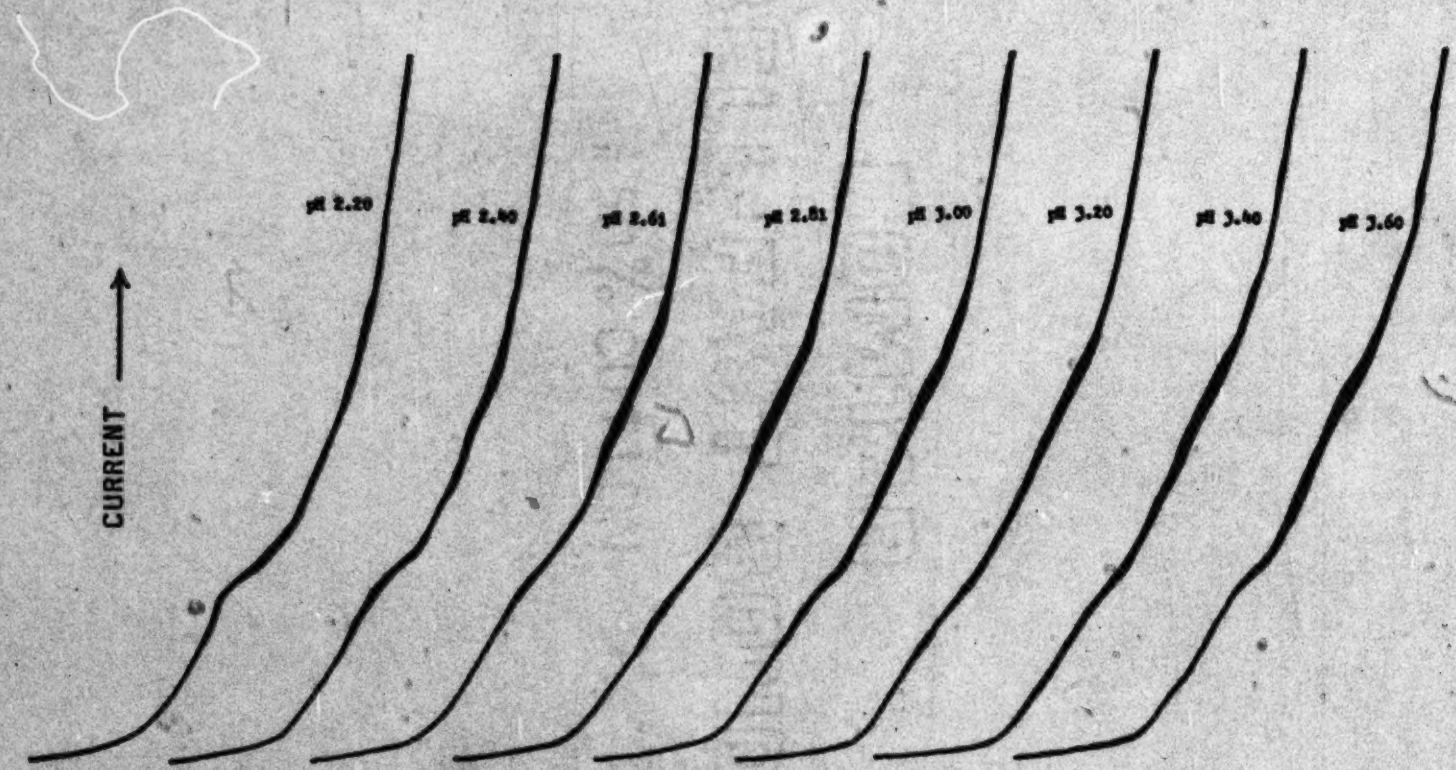
FIGURE 2 CONTINUED: CURRENT-VOLTAGE CURVES OF TETRACYCLINE HYDROCHLORIDE IN BRITTON-ROBINSON BUFFERS.



Each segment begins at -0.90 V. Each additional 0.10 V is represented by ⟵

Concentration = 1.11×10^{-4} M.

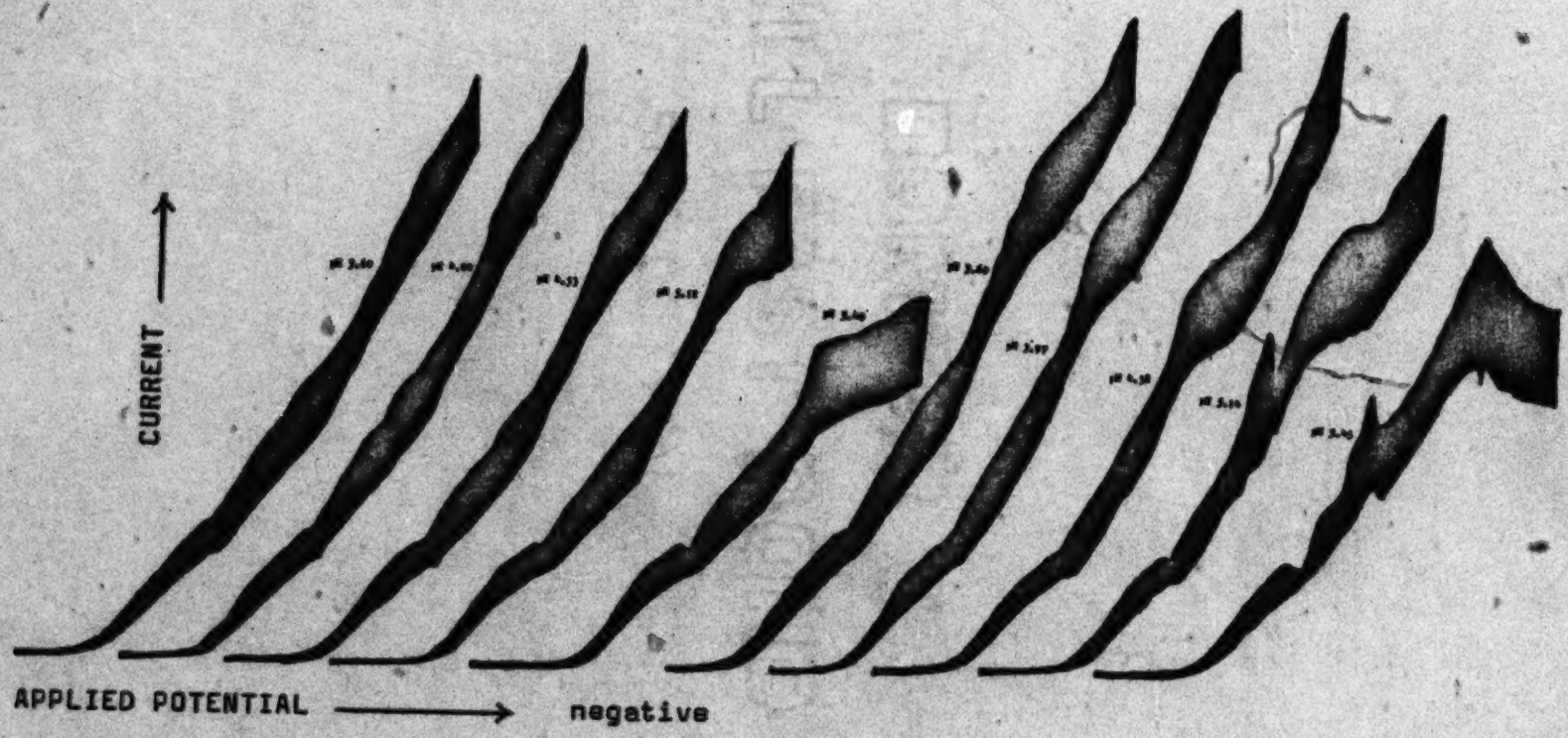
FIGURE 3: CURRENT-VOLTAGE CURVES OF TETRACYCLINE HYDROCHLORIDE IN GLYCINE-HCl BUFFER



Each segment begins at -0.60 V. Each additional 0.10 V is represented by ⟷

Concentration = 1.0×10^{-4} M.

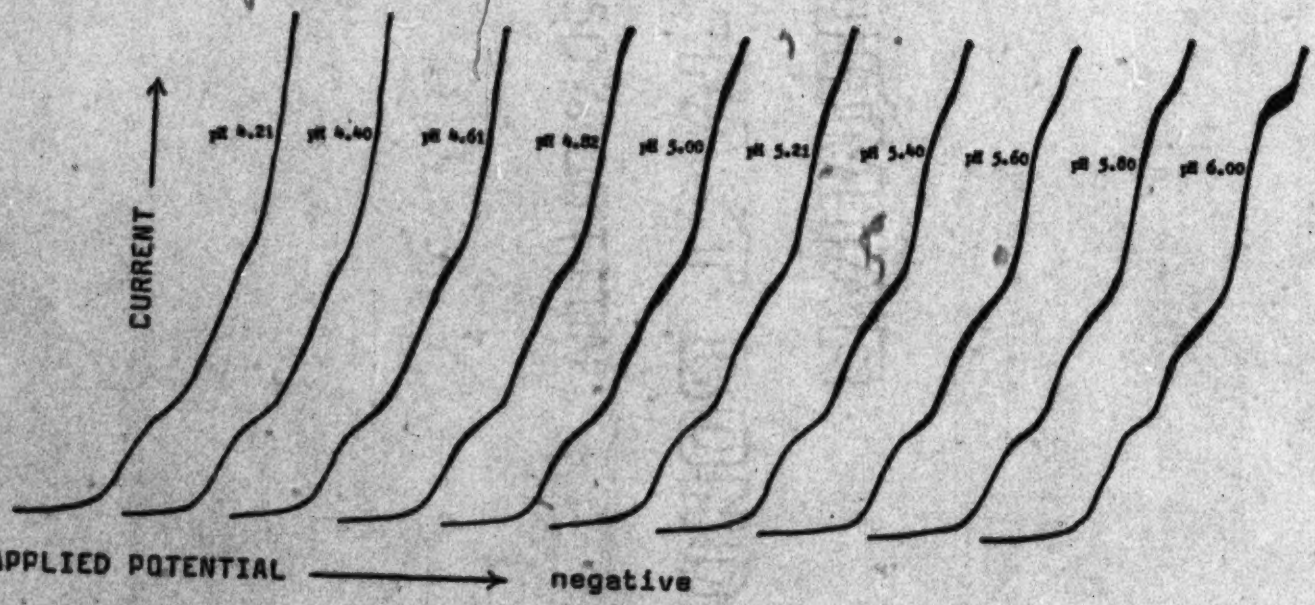
FIGURE 4: CURRENT-VOLTAGE CURVES OF TETRACYCLINE HYDROCHLORIDE IN LITHIUM ACETATE-ACETIC ACID BUFFER (THE FIRST FIVE USING LiCl, THE LAST FIVE USING TETRAETHYL AMMONIUM BROMIDE AS SUPPORTING ELECTROLYTE).



Each segment begins at -0.90 V. Each additional 0.10 V is represented by ⟵

Concentration = 1.0×10^{-4} M.

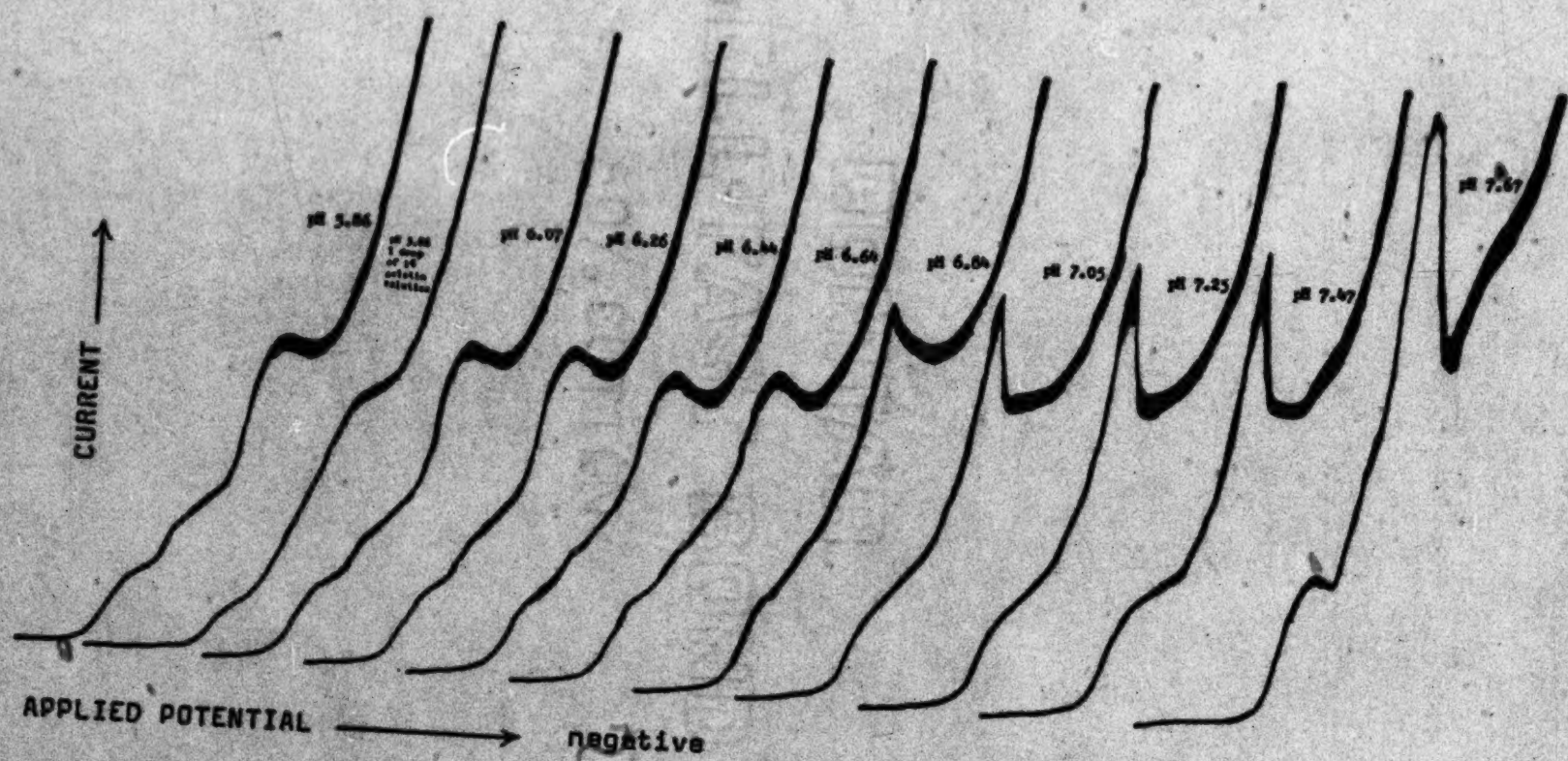
FIGURE 5: CURRENT-VOLTAGE CURVES OF TETRACYCLINE HYDROCHLORIDE IN POTASSIUM ACID PHTHALATE-SODIUM HYDROXIDE BUFFER



Each segment begins at -0.70 V. Each additional 0.10 V is represented by ⟝

Concentration = 1.0×10^{-4} M.

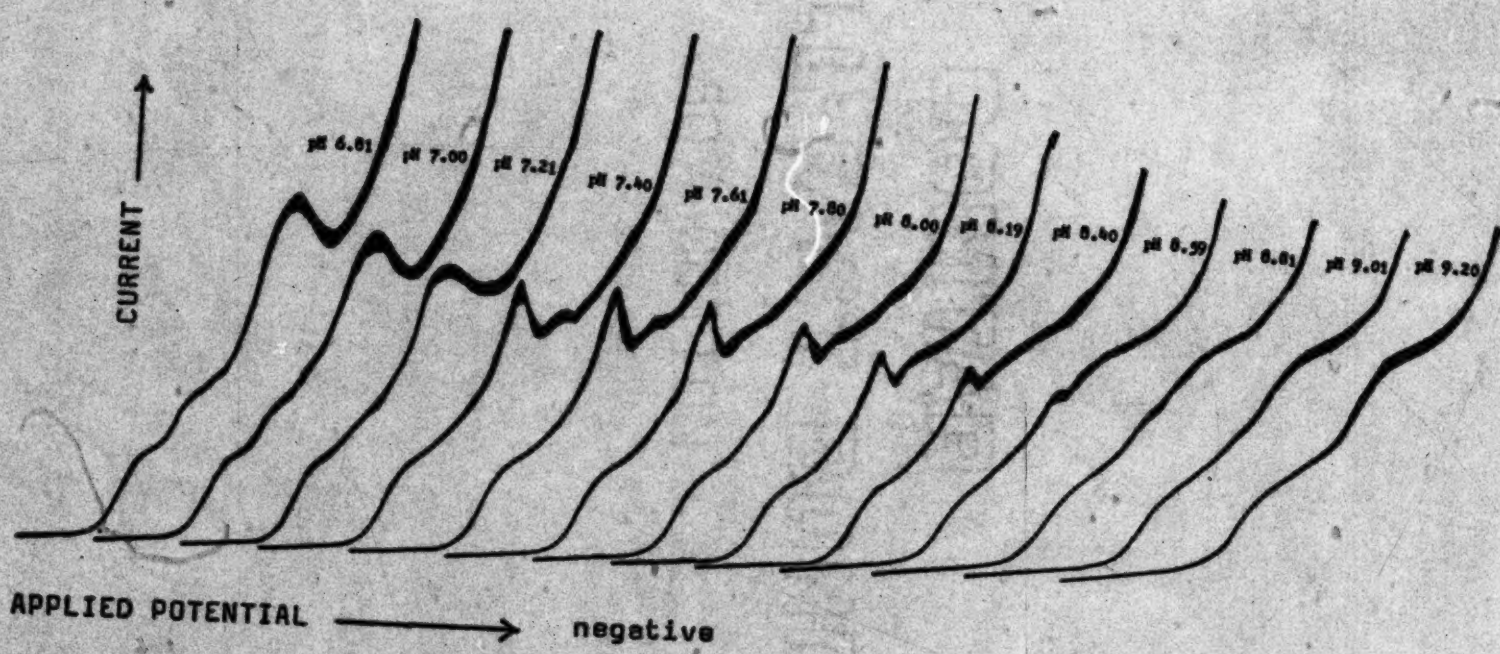
FIGURE 6: CURRENT-VOLTAGE CURVES OF TETRACYCLINE HYDROCHLORIDE IN PHOSPHATE BUFFER



Each segment begins at -0.80 V. Each additional 0.10 V is represented by ⊢⊣

Concentration = 1.0×10^{-4} M.

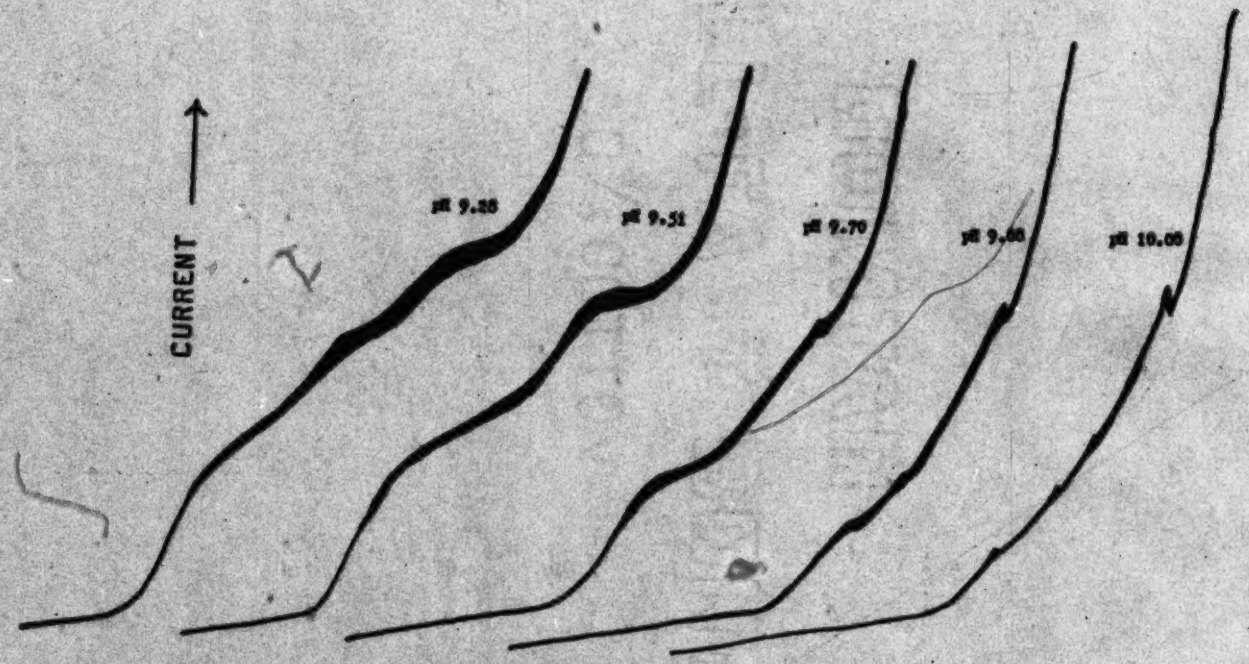
FIGURE 7: CURRENT-VOLTAGE CURVES OF TETRACYCLINE HYDROCHLORIDE IN SODIUM BARBITAL-HYDROCHLORIC ACID BUFFER



Each segment begins at -0.80 V. Each additional 0.10 V is represented by ⊢

Concentration = 1.0×10^{-4} M.

FIGURE 8: CURRENT-VOLTAGE CURVES OF TETRACYCLINE HYDROCHLORIDE IN BORAX-SODIUM HYDROXIDE BUFFER

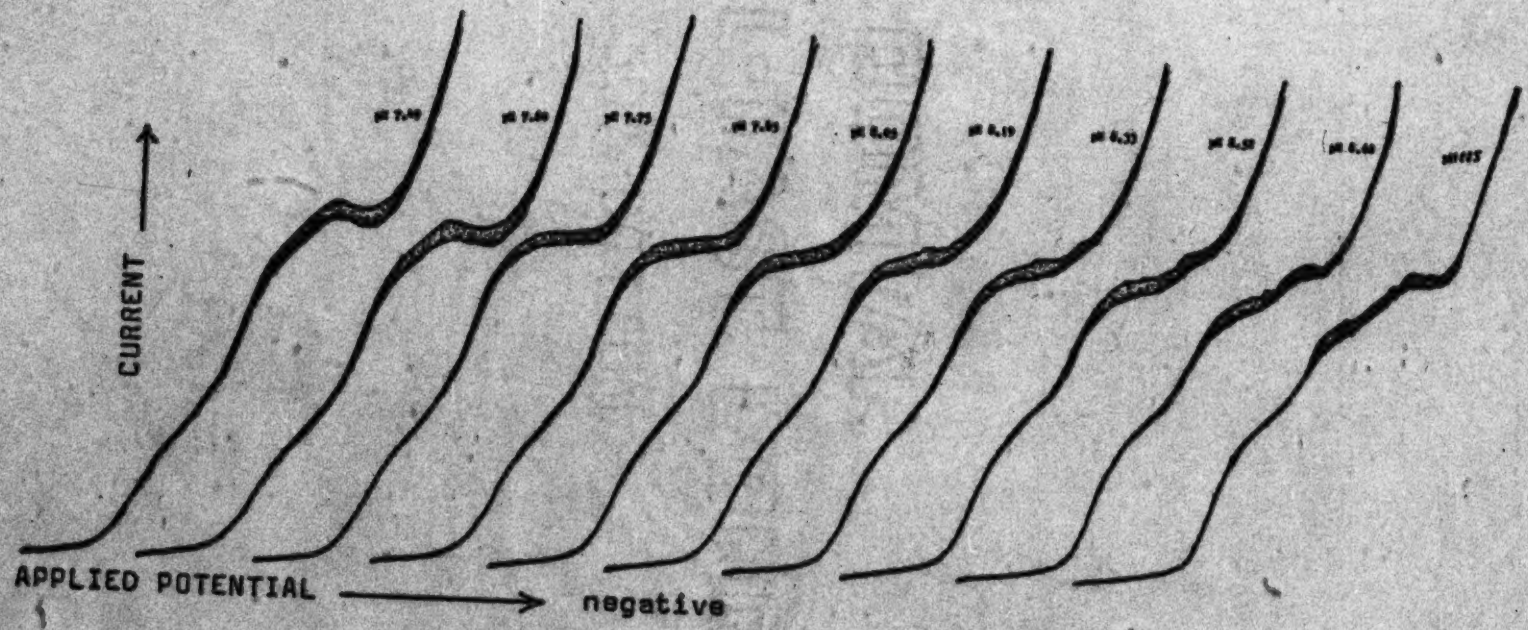


APPLIED POTENTIAL ⟶ negative

Each segment begins at -1.00 V. Each additional 0.10 V is represented by ⟷

Concentration = 1.0×10^{-4} M.

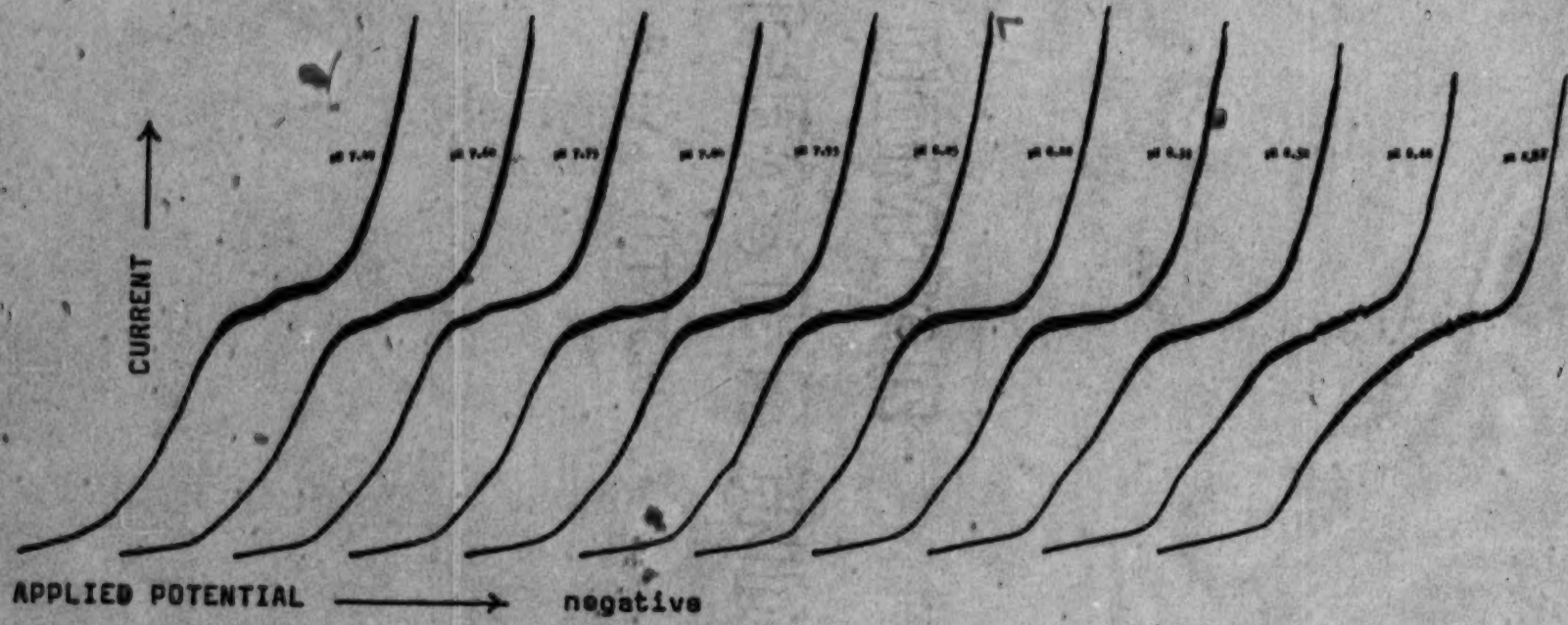
FIGURE 9: CURRENT-VOLTAGE CURVES OF TETRACYCLINE HYDROCHLORIDE IN BORIC ACID-BORAX BUFFER



Each segment begins at -0.90 V. Each additional 0.10 V is represented by ⟵

Concentration = 1.17×10^{-4} M.

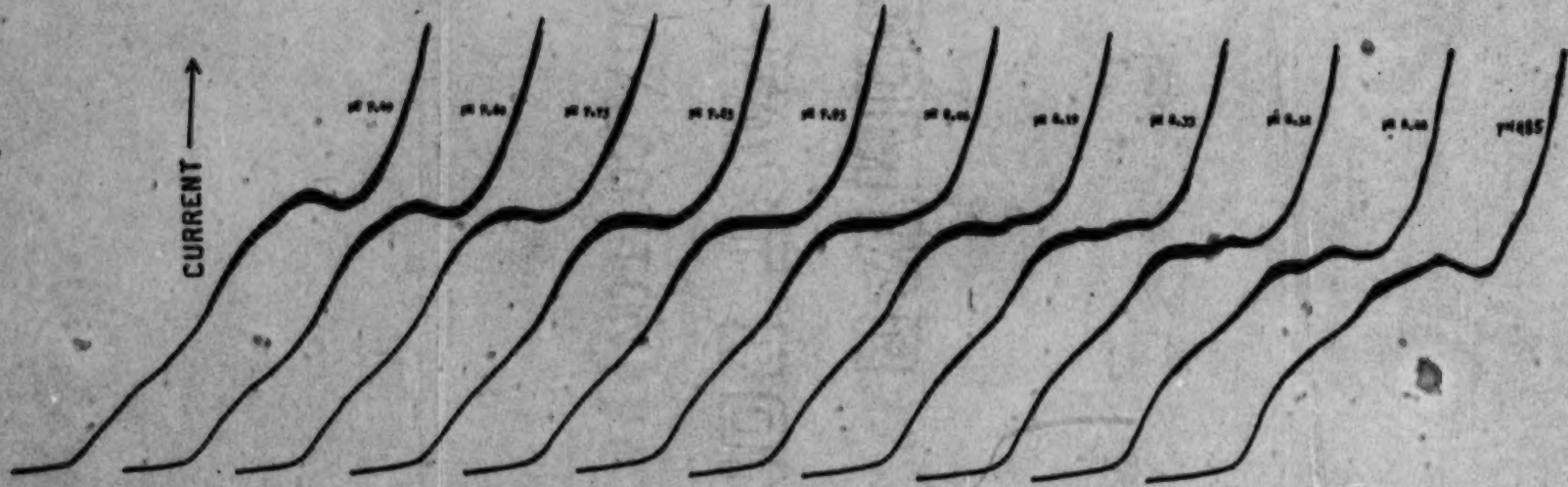
FIGURE 10: CURRENT-VOLTAGE CURVES OF OXYTETRACYCLINE HYDROCHLORIDE IN BORIC ACID-BORAX BUFFER



Each segment begins at -0.90 V. Each additional 0.10 V is represented by ⟼

Concentration = 1.06×10^{-4} M.

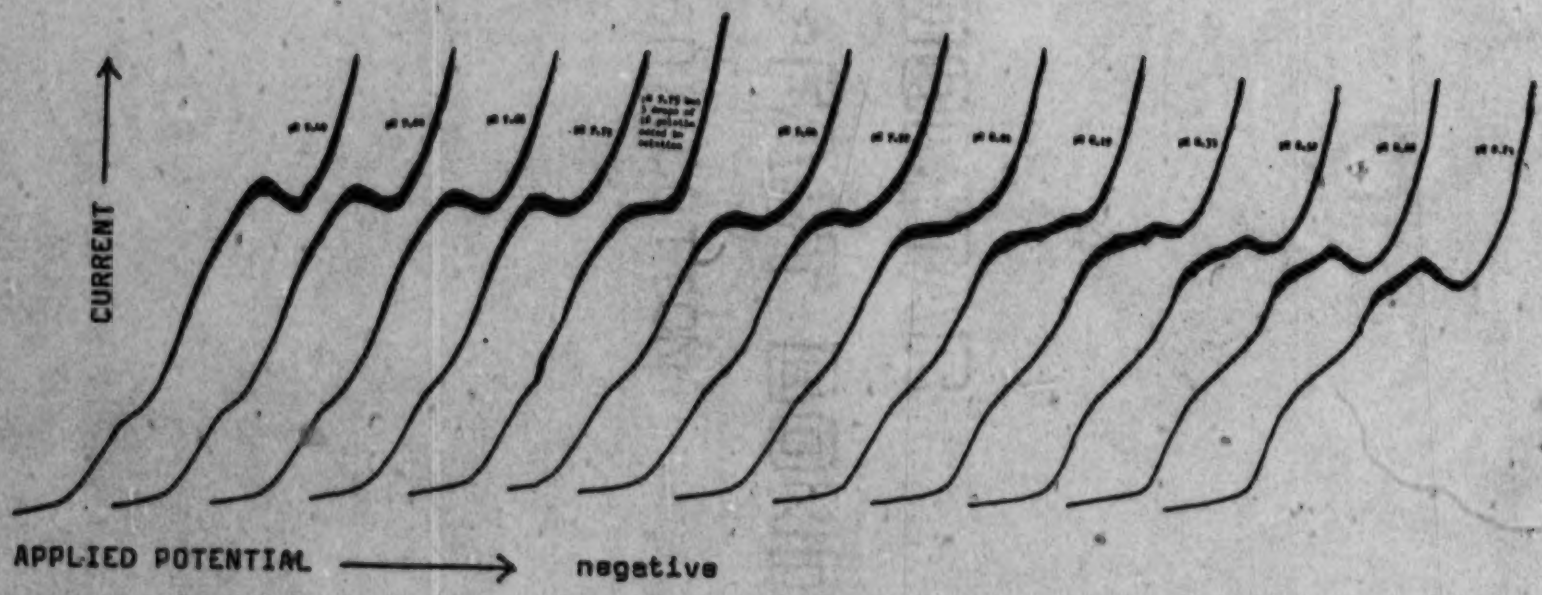
FIGURE 11: CURRENT-VOLTAGE CURVES OF CHLORTETRACYCLINE HYDROCHLORIDE IN BORIC ACID-BORAX BUFFER



Each segment begins at -0.90 V. Each additional 0.10 V is represented by ⟷

Concentration = 9.49×10^{-5} M.

FIGURE 12: CURRENT-VOLTAGE CURVES OF DEMETHYLCHLORTETRACYCLINE HYDROCHLORIDE IN BORIC ACID-BORAX BUFFER

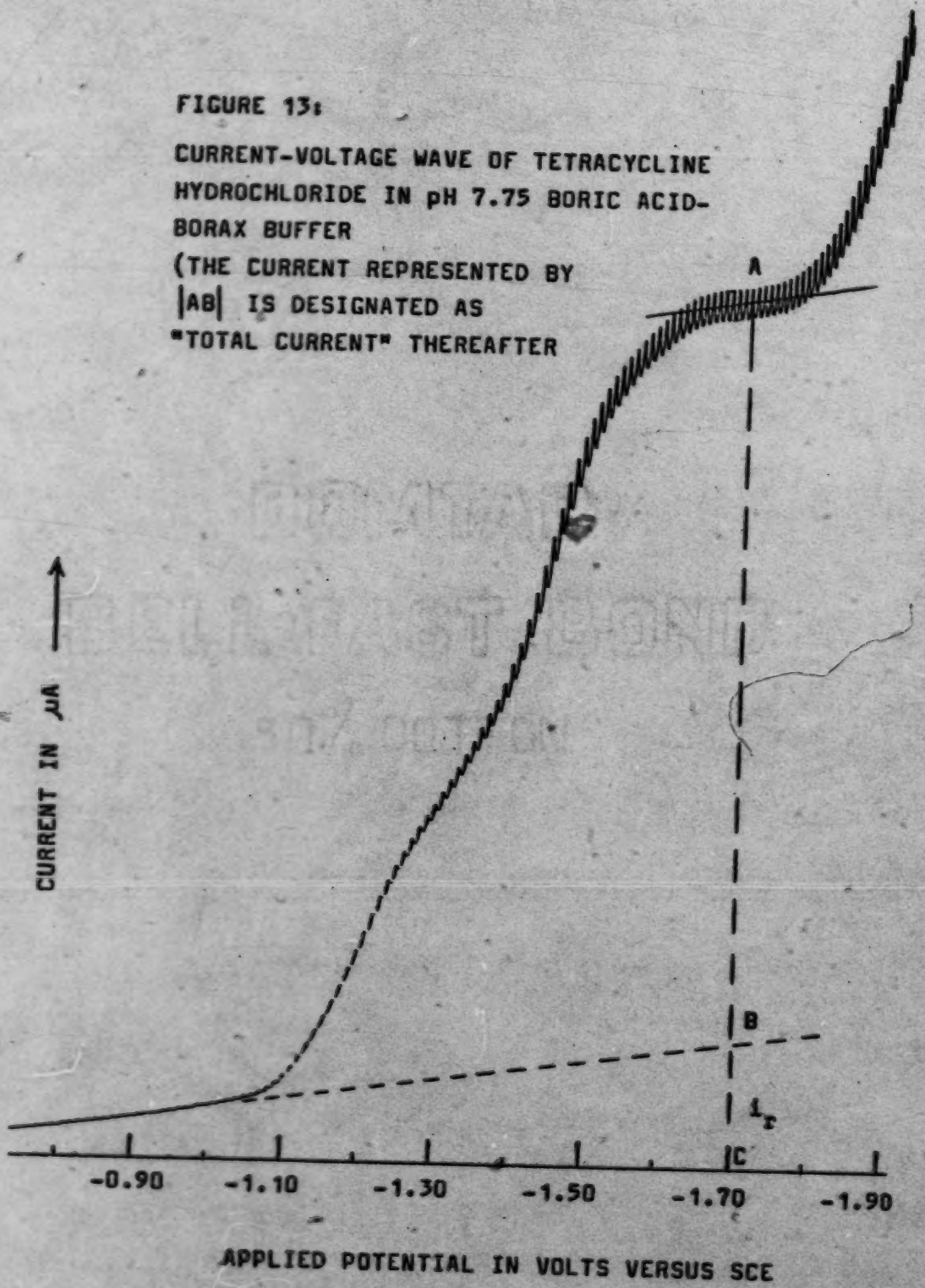


Each segment begins at -0.90 V. Each additional 0.10 V is represented by ⟼

Concentration = 1.05×10^{-4} M.

FIGURE 13:

CURRENT-VOLTAGE WAVE OF TETRACYCLINE HYDROCHLORIDE IN pH 7.75 BORIC ACID-BORAX BUFFER
(THE CURRENT REPRESENTED BY |AB| IS DESIGNATED AS "TOTAL CURRENT" THEREAFTER



were obtained every half hour from the time the solutions were prepared up to five hours. The results on each drug showed that there was no significant change in the shape of the polarograms, and this five hour period of stability was sufficiently long to permit completion of a set of assays.

F. Determination of pH Change before and after Electrolysis

The solutions of each tetracycline salt were prepared using their specified buffer to obtain concentrations of each drug at 0.100 mg and 1.250 mg per 10 ml of solution. It was found that only a 0.05 pH unit maximum decrease from the specified pH occurred under these conditions. Comparisons of the polarograms were made between a control without additional adjustment of the pH and the one with pH adjustment by adding an appropriate amount of borax powder to the specified value. The results showed that even the maximum 0.05 pH unit difference still gave no significant variations in the polarograms. Furthermore, the pH was found to be constant before and after the polarographic electrolysis.

G. Determination of Diffusion Dependency

Actually, polarographic curves can be classified into various types according to the process that governs the value of the limiting current. The most common types of polarographic limiting currents observed in the presence of electroactive species are diffusion currents, kinetic currents, catalytic currents and adsorption currents.

Diffusion currents are governed by the rate of diffusion of the electroactive species toward the surface of the electrode. Kinetic currents are governed by a chemical reaction rate accompanying the electrode process proper and taking place at or near the electrode surface. Catalytic currents are also governed by the chemical reaction rate, but the reaction in this case can be classified as catalytic. Adsorption currents are those that are limited by the coverage of the surface of the electrode, either by the original form of the species being electrolyzed, by an intermediate product, or by the final electrolysis product.

The types of polarographic limiting currents can be distinguished by following the effects of the electroactive species under study and the height of the mercury column. Additional information may be obtained by observing how wave heights are affected by changing the pH, temperature and composition of the system.

An increase of the concentration of the species studied causes diffusion currents and most kinetic currents to increase linearly with increasing concentration. A catalytic current usually reaches a certain limiting value as the concentration is increased, while the adsorption currents are practically independent of the concentration changes.

Generally, increasing the mercury column height results in an increase in the limiting current. Diffusion

currents are a linear function of the square root of the mercury column height, which must be corrected for the back pressure of the solution. In addition, the plot of diffusion current against the square root of the corrected mercury column height must pass through the origin, if the process is completely diffusion dependent. A limiting current that is governed solely by the chemical reaction rate (i.e. a pure kinetic current) does not change with the change of mercury pressure. Thus, a plot of current due to the kinetic mechanism against the square root of the corrected mercury column height or against the corrected mercury column height itself will be linear with a slope of zero. When a plot is made of current against the square root of the corrected mercury column height, if the slope is smaller than that for a process which is entirely diffusion controlled but is greater than zero, this usually indicates that the current is at least partially influenced by kinetic factors. In addition, the extrapolated plot does not pass through the origin. For catalytic currents, various types of $i\text{-}h^n$ (current-corrected height^n) dependencies can be obtained, according to the compound involved and the conditions used. Adsorption currents, in this case, are linearly proportional to the corrected mercury column height and therefore give linear plots of current against the corrected mercury column height.

In discussing the pH and composition of the system,

the following summary can be made. Diffusion- and adsorption-controlled currents are rarely pH dependent and almost always independent of the composition of the system, however, the kinetic and catalytic currents are often a function of pH, since the rate of the chemical process involved is pH dependent. The kind and concentration of the buffer system have also some effect on the kinetic and catalytic currents.

Temperature variation might also give some useful information in helping to delineate the type of current. It is well known that increasing the temperature will cause the diffusion current to increase by about 1.8% per degree Centigrade. Some kinetic currents show a much more pronounced dependence on temperature, but others behave similarly to diffusion currents. Hence, a current whose temperature coefficient is large is most probably a kinetic current. However, a temperature coefficient of about 2% per degree does not allow the exclusion of the possibility that the kinetic current is involved. Adsorption currents have several types of response to temperature change. Some do not change with temperature, some increase as diffusion currents do, and others decrease or even vanish if the temperature is raised high enough. Therefore, only the last type can be used as a diagnostic proof of an adsorption current. It is imperative to know whether the electroreduction process of the respective tetracyclines is

diffusion dependent. Consequently, solutions of each antibiotic in this investigation were prepared at approximately 1×10^{-4} M in the boric acid-borax buffer and at the optimum pH for each tetracycline. For each drug, the height of the mercury column was arbitrarily set at intervals ranging from 56.0 cm to approximately 90.0 cm and the polarograms were obtained at each of the six heights selected. In each instance, the observed mercury height, the mercury flow rate, the drop time, the back pressure, the corrected height of the mercury column and the total current height were either recorded or calculated. By plotting the total current against the square root of the corrected height of the mercury column for each antibiotic, a linear relationship was observed, in each instance. The data for tetracycline hydrochloride is presented in Table II while Figure 14 is the graphic representation. Table III contains the data for oxytetracycline hydrochloride and Figure 15 is the plot of the data. Table IV and Figure 16 represent the data and plot respectively for chlortetracycline hydrochloride and Table V and Figure 17 for demethylchlortetracycline hydrochloride.

In none of the aforementioned figures did the extrapolated line pass through the origin. It is apparent, therefore, that while the reduction process is largely dependent upon diffusion, some other parameter or parameters are also influencing the mechanism. Based on the

Table II Data for Determination of Diffusion Dependency for Tetracycline Hydrochloride

Observed Hg Height (cm)	m in mg/sec	t in sec	Back Pressure $3.1/ m^{1/3} t^{1/3}$	Corrected Hg Height	$\sqrt{\text{Corrected Hg Height}}$	Diffusion Current	Average in μA
56.0	2.5298	3.16	1.551	54.449	7.379	1.4535 1.4505	1.4520
65.0	2.9448	2.72	1.549	63.451	7.966	1.5330 1.5360	1.5345
71.0	3.1798	2.50	1.553	69.447	8.334	1.5960 1.5900	1.5930
78.0	3.5423	2.27	1.547	76.453	8.744	1.6530 1.6590	1.6560
85.0	3.8488	2.09	1.547	83.453	9.135	1.7085 1.7085	1.7085
90.3	4.0713	1.96	1.551	88.749	9.421	1.7520 1.7580	1.7550

(i) All of the above determinations were performed at 25.0° C.

(ii) The solution prepared was at 1.175×10^{-4} M, and all measurements were made at -1.70 V vs SCE.

FIGURE 14:

DETERMINATION OF DIFFUSION DEPENDENCY OF TETRACYCLINE HYDROCHLORIDE (CONCENTRATION OF 1.175×10^{-4} M)

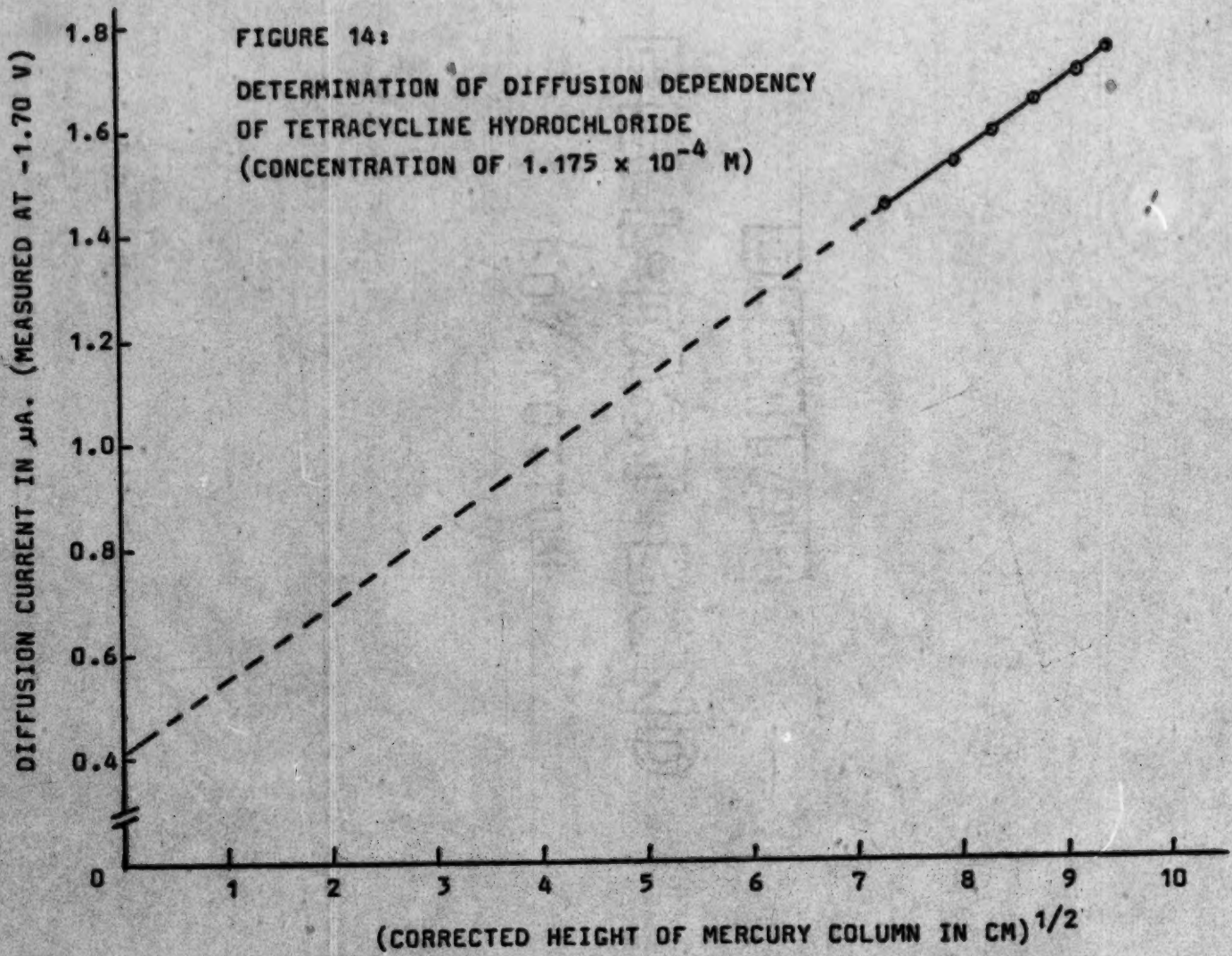


Table III Data for Determination of Diffusion Dependency for Oxytetracycline Hydrochloride

Observed Hg Height (cm)	m in mg/sec	t in sec	Back Pressure $3.1/ m^{1/3} t^{1/3}$	Corrected Hg Height	$\sqrt{\text{Corrected Hg Height}}$	Diffusion Current	Average in μA
56.0	2.5209	2.852	1.606	54.394	7.375	1.1325 1.1295 1.1355	1.1325
65.0	2.9575	2.454	1.601	63.399	7.962	1.1595 1.1640 1.1655	1.1630
71.0	3.2232	2.252	1.601	69.399	8.331	1.1790 1.1880 1.1835	1.1835
78.0	3.5494	2.054	1.599	76.401	8.741	1.2015 1.2045 1.2030	1.2030
85.0	3.9010	1.878	1.596	83.404	9.133	1.2270 1.2300 1.2345	1.2305
90.0	4.0922	1.772	1.602	88.398	9.402	1.2465 1.2435 1.2480	1.2460

(i) All of the above determinations were performed at 25.0° C.

(ii) The solution prepared was at 1.107×10^{-4} M, and all measurements were made at -1.60 V vs SCE.

FIGURE 15:

DETERMINATION OF DIFFUSION DEPENDENCY
OF OXYTETRACYCLINE HYDROCHLORIDE
(CONCENTRATION OF 1.107×10^{-4} M)

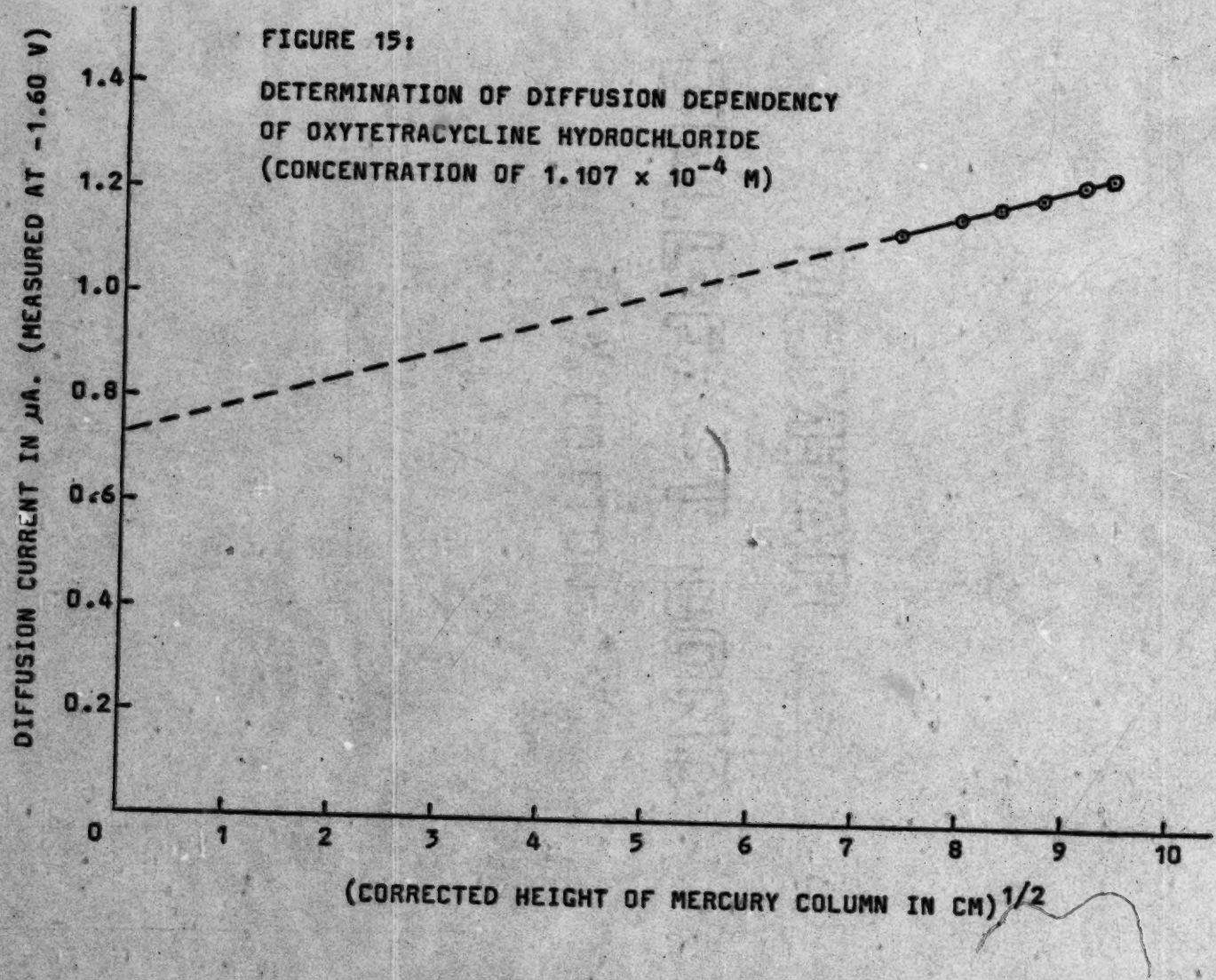


Table IV Data for Determination of Diffusion Dependency for Chlortetracycline Hydrochloride

Observed Hg Height (cm)	m in mg/sec	t in sec	Back Pressure 3.1/ $m^{1/3}$ $t^{1/3}$	Corrected Hg Height	$\sqrt{\text{Corrected Hg Height}}$	Diffusion Current	Average in μA
56.0	2.5192	2.86	1.605	54.395	7.375	1.4685 1.4655 1.4655	1.4665
65.0	2.9319	2.49	1.598	63.402	7.963	1.5585 1.5570 1.5570	1.5575
71.0	3.1811	2.30	1.597	69.403	8.331	1.6185 1.6155 1.6155	1.6165
78.0	3.5482	2.05	1.600	76.400	8.741	1.6800 1.6785 1.6800	1.6795
85.0	3.8347	1.90	1.599	83.401	9.132	1.7415 1.7385 1.7400	1.7400
90.3	4.0629	1.80	1.597	88.703	9.418	1.7820 1.7850 1.7850	1.7840

(i) All of the above determinations were performed at 25.0° C.

(ii) The solution prepared was at 1.232×10^{-4} M, and all measurements were made at -1.66 V vs SCE.

FIGURE 16:

DETERMINATION OF DIFFUSION DEPENDENCY OF CHLORTETRACYCLINE HYDROCHLORIDE (CONCENTRATION OF 1.232×10^{-4} M)

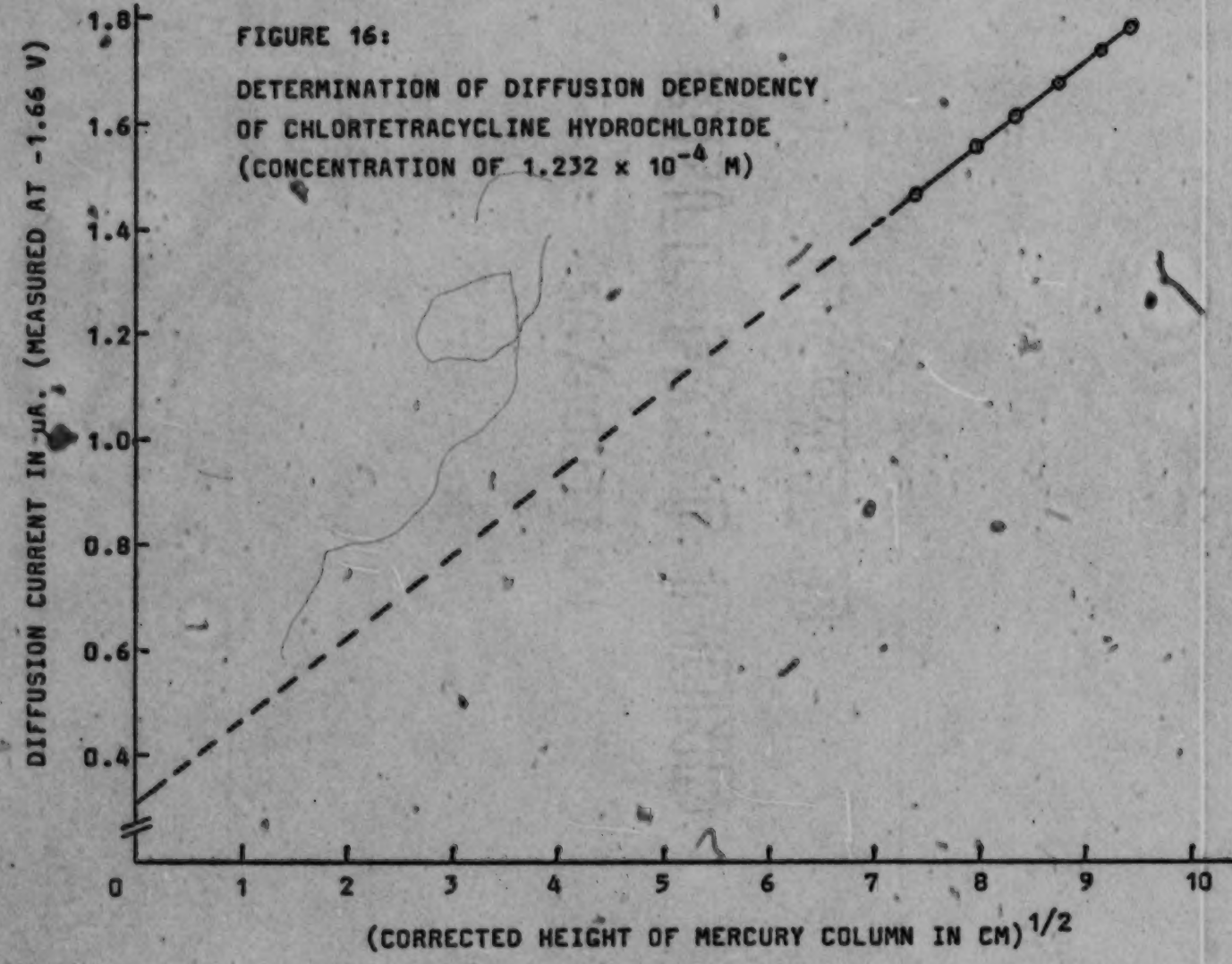


Table V Data for Determination of Diffusion Dependency for Demethylchlortetracycline Hydrochloride

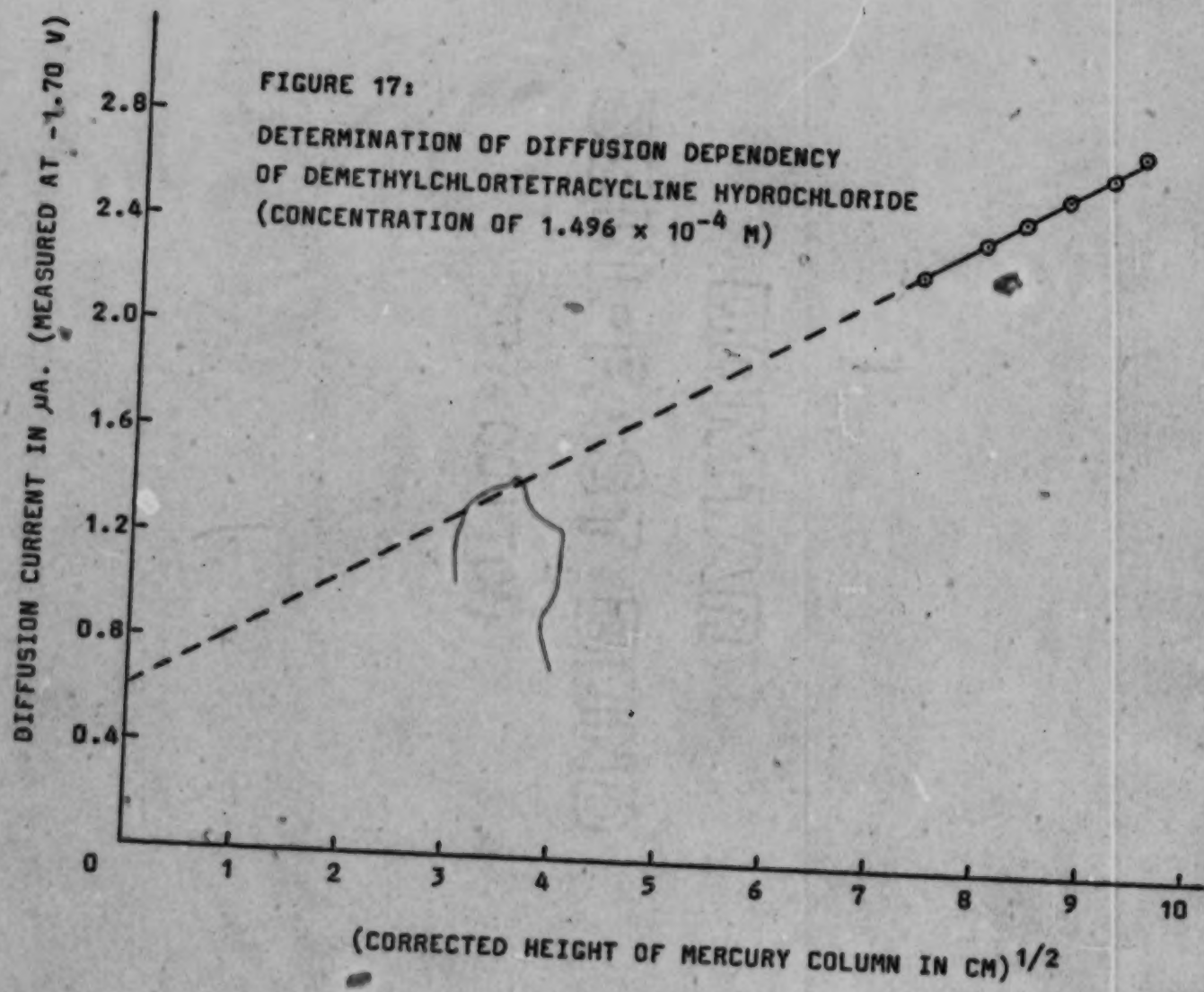
Observed Hg Height (cm)	m in mg/sec	t in sec	Back Pressure $3.1/ m^{1/3} t^{1/3}$	Corrected Hg Height	$\sqrt{\text{Corrected Hg Height}}$	Diffusion Current	Average in μA
56.0	2.4878	2.85	1.614	54.386	7.375	2.2560 2.2530 2.2530	2.2540
65.0	2.8959	2.45	1.613	63.387	7.962	2.3850 2.3850 2.3835	2.3845
71.0	3.1272	2.25	1.618	69.382	8.330	2.4690 2.4690 2.4690	2.4690
78.0	3.4835	2.02	1.618	76.382	8.740	2.5620 2.5575 2.5620	2.5605
85.0	3.8024	1.85	1.618	83.382	9.131	2.6535 2.6535 2.6520	2.6530
90.3	4.0038	1.74	1.623	88.677	9.417	2.7450 2.7420 2.7450	2.7440

(i) All of the above determinations were performed at 25.0° C.

(ii) The solution prepared was at 1.496×10^{-4} M, and all measurements were made at -1.70 V vs SCE.

FIGURE 17:

DETERMINATION OF DIFFUSION DEPENDENCY
OF DEMETHYLCHLORTETRACYCLINE HYDROCHLORIDE
(CONCENTRATION OF 1.496×10^{-4} M)



experience and report of Zuman (70), it could be suggested that kinetic factors play a significant part in the reduction process of the tetracyclines. This influence appears to be more pronounced for oxytetracycline and demethylchlortetracycline than for the other two antibiotics.

Furthermore, from the experience and data obtained in the foregoing experiments, two additional important conclusions could be drawn. Firstly, the mercury flow rate and the dropping time varied if they were measured at different potentials, even though the mercury height remained constant (eg. oxytetracycline·HCl and tetracycline·HCl were measured at -1.60 and -1.70 V, respectively). In other words, the back pressure of mercury varied with the applied potential. Secondly, the addition of gelatin solution also influenced the mercury flow rate and dropping time as is evident from the comparisons of Table II and Table V (for tetracycline hydrochloride and demethylchlortetracycline hydrochloride, respectively).

H. Preparation of Calibration Curves

(i) Tetracycline Hydrochloride:

The standard solutions of tetracycline hydrochloride were prepared in the boric acid-borax buffer system at pH 7.75. The concentrations were 0.100 mg, 0.250 mg, 0.375 mg, 0.500 mg, 0.750 mg, 1.000 mg and 1.250 mg per 10 ml of solution. Five separate determinations were polarographed for each concentration and the data recorded in Table VI.

The average total current measured at -1.70 V versus SCE was plotted against each concentration, and a linear relationship was obtained as shown in Figure 18 (page 78). If this line could have been extrapolated to pass through the origin, one would say that the electroreduction process is purely diffusion dependent. However, since the extrapolation of the calibration curve does not pass through the origin, it would appear that some kinetic characteristics are influencing the process at the DME.

Due to the inherent residual current, the lowest concentration that could be measured was 0.050 mg/ml (equivalent to 1.039×10^{-5} M). For practical purposes, the concentration range that permitted easy measurements with confidence and accuracy was selected between 0.100 mg/10 ml and 1.250 mg/10 ml (equivalent to 2.079×10^{-5} M and 2.599×10^{-4} M, respectively). In comparing the polarograms of the standard solutions one with another, it was observed that an increase in the concentration of drug resulted in the center point of the limiting current plateau being shifted to a more negative value. Although it is not possible to accurately determine the $E_{1/2}$ value for either of the waves, there is no doubt that they are similarly shifted. In a recent publication, Bond (71) has stated that in all probability such a shift implies that the reduction process is irreversible. The total current, however, was always measured at the same specified potential, in each instance.

Table VI Data for Calibration Curve of Tetracycline Hydrochloride

Conc. of Drug (mg/10 ml)	Total Current Measured at -1.70 V vs SCE in μA					
	1	2	3	4	5	Average
0.100	0.3156	0.3240	0.3234	0.3240	0.3240	0.3222
0.250	0.7320	0.7268	0.7332	0.7203	0.7215	0.7268
0.375	1.0860	1.0808	1.0816	1.0760	1.0796	1.0808
0.500	1.3750	1.3780	1.3800	1.3780	1.3705	1.3763
0.750	2.0213	2.0175	2.0115	2.0108	2.0100	2.0142
1.000	2.6370	2.6445	2.6430	2.6475	2.6610	2.6466
1.250	3.2560	3.2560	3.2540	3.2540	3.2560	3.2552

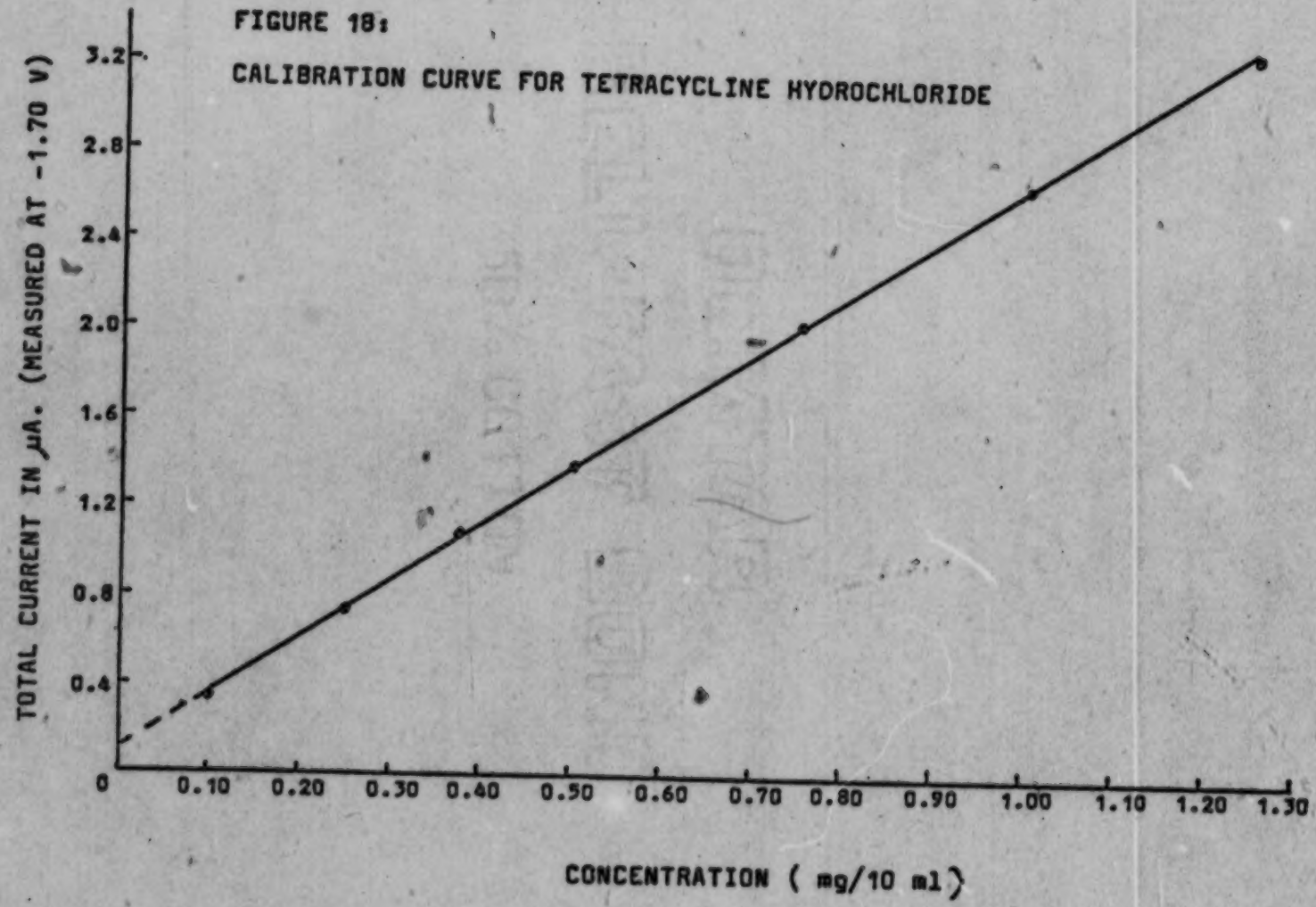
(i) Potency of tetracycline hydrochloride reference standard was determined as 100.10% (see Table I).

(ii) The current scale was set at 0.0150 μA/mm .

(iii) All of the above determinations were performed at 25.0° C.

FIGURE 18:

CALIBRATION CURVE FOR TETRACYCLINE HYDROCHLORIDE



(ii) Oxytetracycline Hydrochloride:

The standard solutions of oxytetracycline hydrochloride were prepared in boric acid-borax buffer at pH 8.20. The concentrations were identical to those employed for tetracycline hydrochloride. The polarograms were obtained in the same manner as previously described. The data are given in Table VII and the calibration curve appears in Figure 19. Although the same tendency for the limiting current plateau of more concentrated solutions to shift to more negative values was noted, all total current measurements were made at -1.60 V. Figure 19 demonstrates that a good linear relationship was obtained when concentrations were plotted against total currents. Again, the lowest concentration that could be measured was about 0.050 mg/10 ml (1.006×10^{-5} M), however, the concentration was chosen between 0.100 mg/10 ml and 1.250 mg/10 ml (2.012×10^{-5} M to 2.516×10^{-4} M, respectively).

(iii) Chlortetracycline Hydrochloride:

The standard solutions of chlortetracycline hydrochloride were prepared in boric acid-borax buffer at pH 7.95 . The same concentration range was selected and the polarograms were determined in the same manner. Despite the aforementioned tendency of increased concentration causing a negative shift on the limiting current plateau, the total currents for this antibiotic were all measured at -1.66 V. The data for five determinations are presented

Table VII Data for Calibration Curve of Oxytetracycline Hydrochloride

Conc. of Drug (mg/10 ml)	Total Current Measured at -1.60 V vs SCE in μA					
	1	2	3	4	5	Average
0.100	0.2700	0.2718	0.2706	0.2694	0.2730	0.2710
0.250	0.5829	0.5844	0.5850	0.5838	0.5886	0.5849
0.375	0.8304	0.8328	0.8320	0.8304	0.8312	0.8314
0.500	1.0708	1.0696	1.0716	1.0696	1.0736	1.0710
0.750	1.5640	1.5580	1.5710	1.5680	1.5640	1.5650
1.000	2.0610	2.0610	2.0595	2.0595	2.0588	2.0600
1.250	2.4915	2.4900	2.4885	2.4915	2.4908	2.4905

(i) Potency of oxytetracycline hydrochloride reference standard was determined as 96.81% (see Table I)

(ii) The current sensitivity scale was set at 0.0150 μA/mm .

(iii) All of the above determinations were performed at 25.0° C.

FIGURE 19:
CALIBRATION CURVE FOR OXYTETRACYCLINE HYDROCHLORIDE

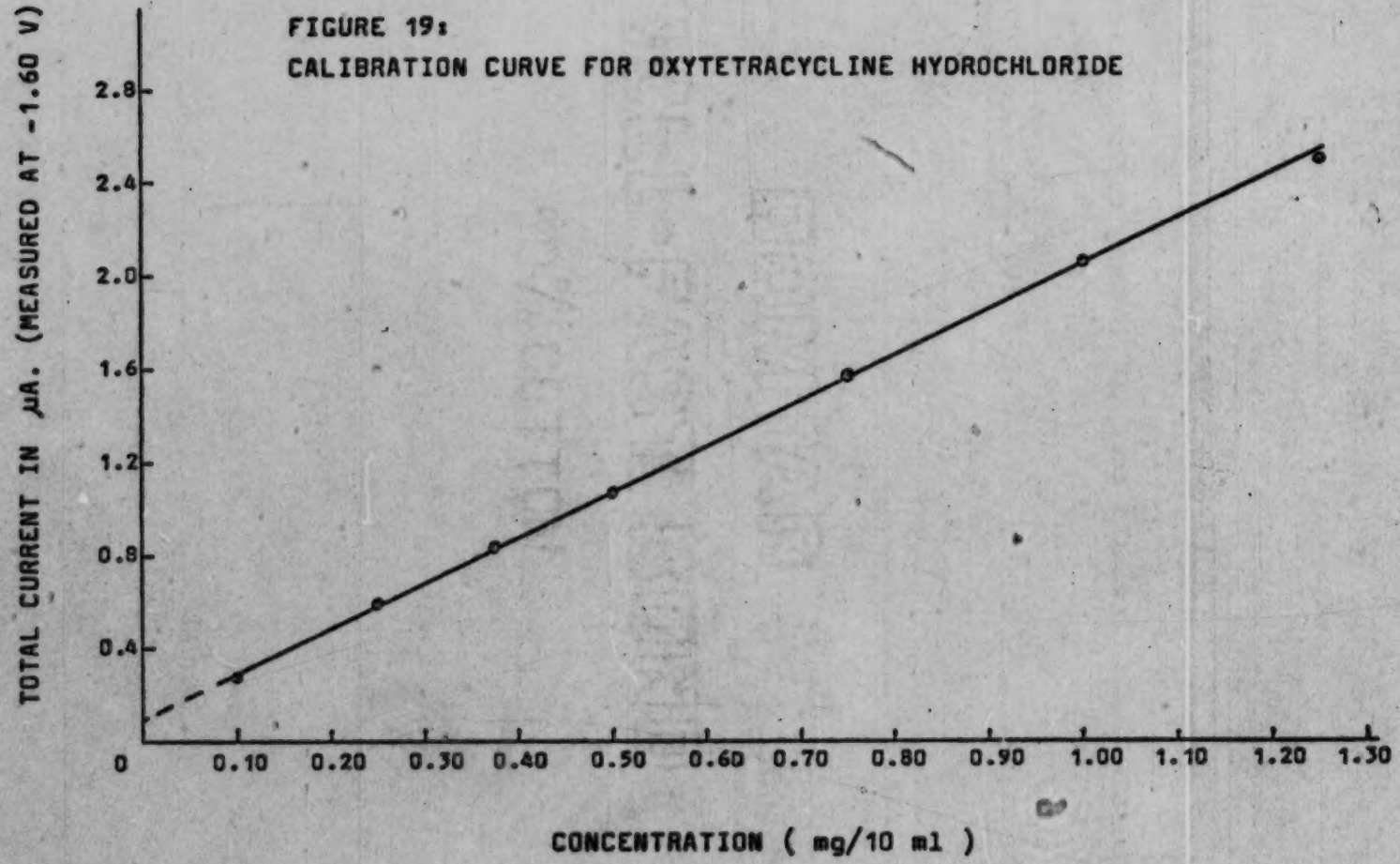


Table VIII Data for Calibration Curve of Chlortetracycline Hydrochloride

Conc. of Drug (mg/10 ml)	Total Current Measured at -1.66 V vs SCE in μA					Average
	1	2	3	4	5	
0.100	0.3210	0.3300	0.3075	0.3240	0.3210	0.3207
0.250	0.7110	0.6975	0.7005	0.6990	0.6900	0.6996
0.375	0.9900	0.9885	0.9825	0.9870	0.9750	0.9846
0.500	1.2705	1.2765	1.2705	1.2750	1.2795	1.2744
0.750	1.8105	1.8075	1.8135	1.8135	1.8075	1.8105
1.000	2.3715	2.3670	2.3625	2.3685	2.3674	2.3674
1.250	2.9010	2.9040	2.9048	2.9175	2.8890	2.9033

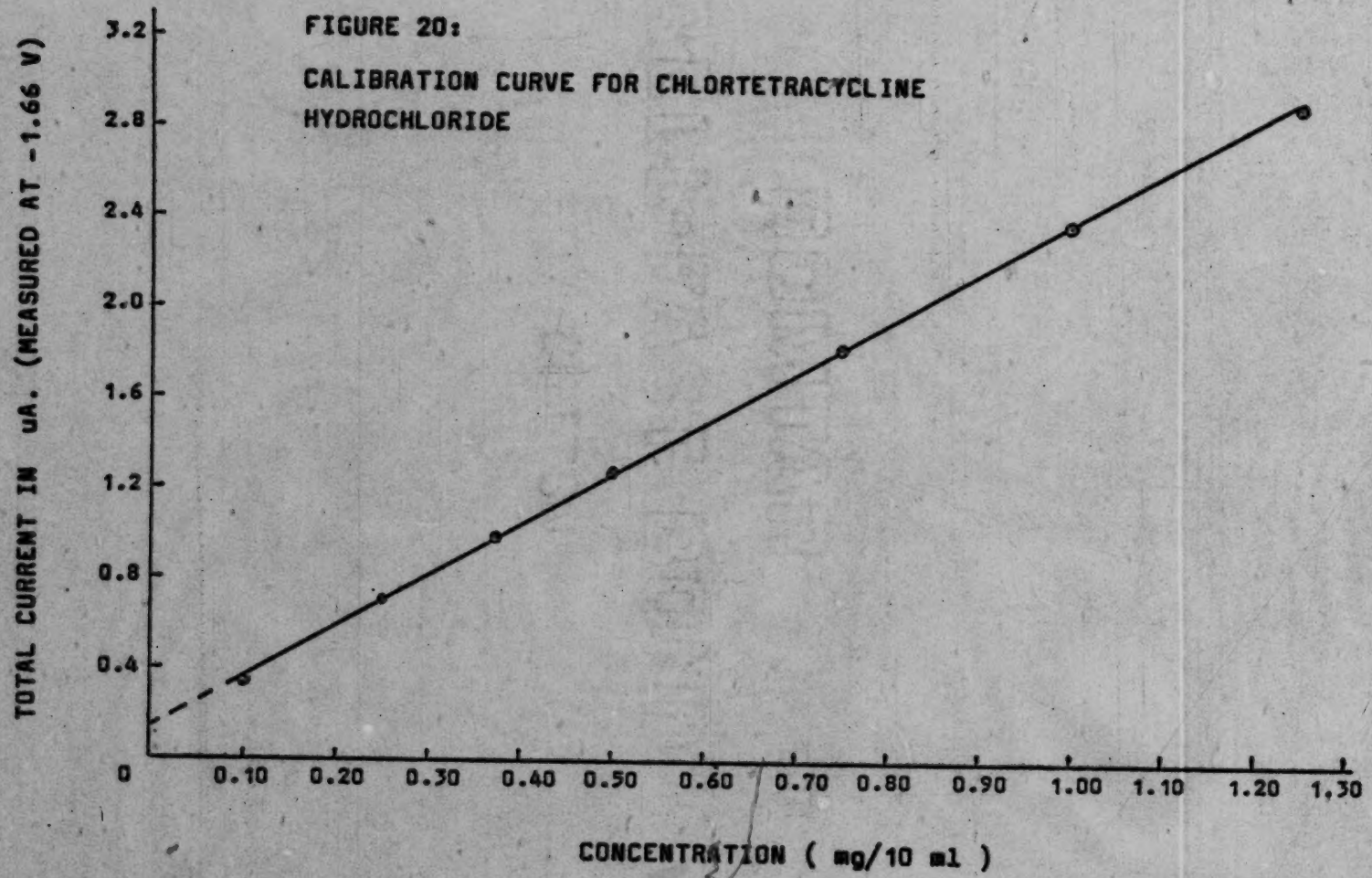
(i) Potency of chlortetracycline hydrochloride reference standard was determined as 97.90% (see Table I).

(ii) The current sensitivity scale was set at 0.0150 μA/mm .

(iii) All of the above determinations were performed at 25.0° C.

FIGURE 20:

CALIBRATION CURVE FOR CHLORTETRACYCLINE HYDROCHLORIDE



in Table VIII and the calibration curve appears in Figure 20. The same considerations were applied to the concentration range, when preparing the calibration curve. The lowest limit was 0.100 mg/10 ml and the upper 1.250 mg/10 ml (1.940×10^{-5} M and 2.426×10^{-4} M, respectively).

(iv) Demethylchlortetracycline Hydrochloride:

The standard solutions of demethylchlortetracycline hydrochloride were prepared in boric acid-borax buffer at pH 7.75. The same concentrations per 10 ml of solution were employed as stated previously for the other tetracyclines. For this drug, however, a maximum suppressor was necessary to eliminate the maxima which appeared as rounded humps on the limiting current plateau (Figure 12). Experimentation showed that for each 25.0 ml solution, the addition of 0.125 ml of freshly prepared 1% gelatin solution was adequate for the purpose. A calibrated dropper was employed to make the addition. The shifting of the limiting current plateau with increased concentration of depolarizer was again evident. However, all total currents were measured at -1.70 V. The data for five consecutive determinations are given in Table IX and the calibration curve for this drug appears in Figure 21. Once again, a linear relationship existed between the concentration range of 0.100 mg/10 ml and 1.250 mg/10 ml (1.995×10^{-5} M and 2.493×10^{-4} M, respectively) and the corresponding total current.

Due to some differences in structure, tetracycline

Table IX Data for Calibration Curve of Demethylchlortetracycline Hydrochloride

Conc. of Drug (mg/10 ml)	Total Current Measured at -1.70 V vs SCE in μA					Average
	1	2	3	4	5	
0.100	0.4260	0.4302	0.4356	0.4296	0.4200	0.4283
0.250	0.8994	0.9006	0.9030	0.9084	0.9048	0.9032
0.375	1.2520	1.2592	1.2608	1.2616	1.2608	1.2589
0.500	1.6615	1.6650	1.6600	1.6615	1.6640	1.6624
0.750	2.3715	2.3670	2.3700	2.3730	2.3775	2.3718
1.000	3.1200	3.1260	3.1120	3.1160	3.1220	3.1192
1.250	3.6940	3.7070	3.7080	3.7040	3.7220	3.7070

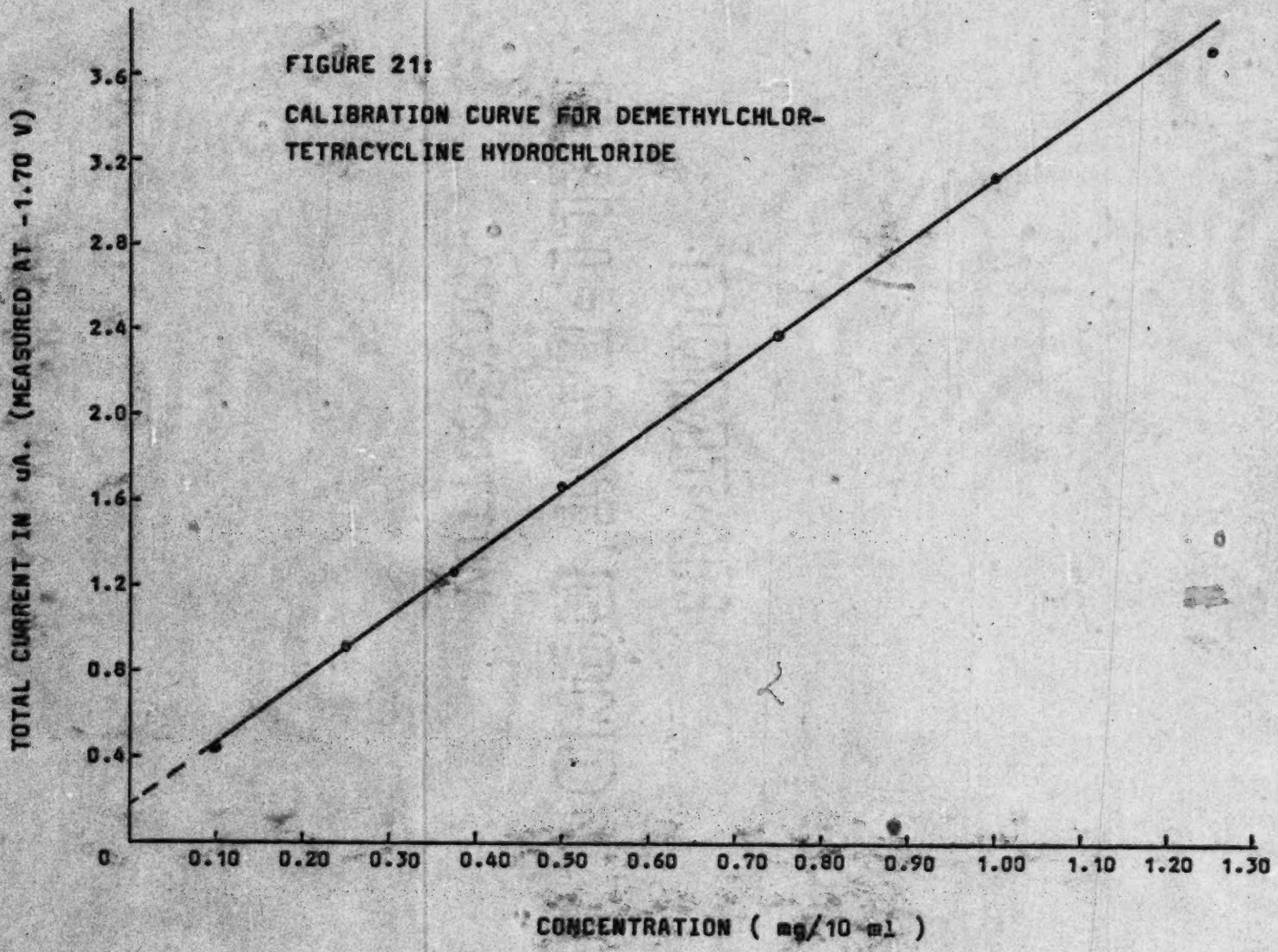
(i) Potency of demethylchlortetracycline hydrochloride reference standard was determined as 100.61% (see Table I)

(ii) The current sensitivity scale was set at 0.0150 μA/mm .

(iii) All of the above determinations were performed at 25.0° C.

FIGURE 21:

CALIBRATION CURVE FOR DEMETHYLCHLOR-TETRACYCLINE HYDROCHLORIDE



hydrochloride and demethylchlortetracycline hydrochloride displayed some different characteristics in their polarograms. The former did not reveal any maximum under the conditions stated previously, while the latter needed some maximum suppressor. Perhaps it could be implied from Heyrovsky's study (72) that the greater the distance from the electrocapillary maximum, the greater the tendency for the occurrence of maxima. Naturally no maximum could be observed when the reduction of the substance took place at a potential corresponding to the electrocapillary maximum. In order to suppress maxima, therefore, the technique is to shift the electrocapillary maximum toward the reduction potential of the species under study. This may be done by the addition of either non-capillary-active ions or capillary-active electrolytes and non-electrolytes to the solution. Thus,negative maxima may be suppressed by the addition of multivalent cations, and positive maxima suppressed by multivalent anions. Almost all maxima are suppressed by surface-active agents, such as gelatin.

The addition of 0.125 ml of 1% gelatin solution in 25.0 ml of solution mentioned above gave a final concentration of 0.005% w/v, which was quite acceptable. In considering the dilution factor, theoretically, the actual concentration of demethylchlortetracycline hydrochloride had to be corrected by multiplying by the factor of 1.005 from (25.0 + 0.125)/ 25.0 . However, since the procedures

used for standard solutions and for commercial products were exactly the same, thus making everything relative, the dilution factor thus could be omitted.

I. Quantitative Assay of Pharmaceutical Dosage Forms

As shown previously in the Experimental Section, numerous procedures for special products were established by modification of the two general methods of analysis. Using these procedures, a large variety of pharmaceutical dosage forms have been assayed for their antibiotic content. Consideration of some of the physicochemical properties of these compounds is important.

Firstly, in the solubility index, the following information was obtained:

(a) Tetracycline: 1 gm of the free base requires 2500 ml of water to dissolve, but 1 gm of its hydrochloride salt requires only 10 ml of water to dissolve (i.e. concentration of 1000 mg/10 ml when saturated).

(b) Oxytetracycline: The free base is only very slightly soluble in water, but 1 gm of its hydrochloride salt dissolve in 2 ml of water (i.e. concentration of 5000 mg/10 ml when saturated).

(c) Chlortetracycline: 1 gm of its hydrochloride salt requires 75 ml of water to dissolve (i.e. concentration of about 133 mg/10 ml when saturated).

(d) Demethylchlortetracycline: 1 gm of its hydrochloride salt dissolves in about 90 ml of water (i.e. concentration

of about 111 mg/10 ml when saturated).

Although the chosen "solvent" for the various hydrochloride salts of tetracycline antibiotics was not pure water but a boric acid-borax buffer solution, there was still no solubility problem in preparing the solutions for both standards and samples to be assayed. In some instances where the tetracyclines existed as free base forms, the addition of appropriate amounts of 3 M hydrochloric acid were made to convert them to the more soluble forms. The pH of the solution was then adjusted to the optimum value for that particular tetracycline by the addition of borax powder.

Secondly, in reviewing the stability of the tetracyclines, it was found that they can undergo a number of facile chemical conversions under a variety of mild conditions of temperature and pH, with often interesting differences between the various members of the group. McCormick *et al.*(73) have reported that epimerization of tetracyclines at the 4-position occurs readily and generally in aqueous solution in the pH range of 2 to 6; the rate of reaction being affected by ions such as phosphate and citrate (74), polyvalent cations (75), and substances such as urea (76). Tetracycline is relatively stable in acid solutions having a pH higher than 2, but it is quickly converted to anhydrotetracycline in more concentrated acid. This phenomenon is more pronounced upon heating. Chlor-

tetracycline undergoes similar reactions under these conditions. For oxytetracycline, however, the anhydro-compound is not usually found since it is readily converted into a mixture of α and β epimeric apoxytetracyclines. In alkaline solution, the tetracyclines undergo isomerization with a concomitant remarkable loss in their potencies. Chlortetracycline, which is particularly susceptible, undergoes a cleavage between carbon atoms 11 and 11a with a resulting new ring formation to give iso-chlortetracycline on heating even at pH 7.5. Interestingly, demethylchlortetracycline differs from chlortetracycline only by the absence of the methyl group on carbon 6, but the absence of this methyl group enhances profoundly the stability of the ring C to both acid and alkali.

The study of the photochemistry of tetracyclines was reviewed and considered in this investigation. With the existence of the carbonyl group, all of the tetracyclines must be photosensitive, since the non-bonding electrons on O can be excited by light to π-orbital and thus hydrogen abstraction can occur. Due to the hydrogen abstraction, the production of free radicals is possible which results in dimer or polymer formation. Therefore, careful protection from light whenever possible was advisable. Plastic or acrylic products are usually transparent to visible and only to some extent to UV light, therefore, these materials can be used to build a vessel which contains the circula-

ting water around the polarographic cell. The advantages of this design need no further elaboration.

Finally, both the free base and the hydrochloride salt of each tetracycline have been determined. It has been established that under the same conditions, both forms give identical waves when differences in molecular weight has been considered. Therefore, attention has to be paid to the manner of indicating labelled potency. Caplis (page 55 in the reference 62) stated that the reduction step of the first polarographic wave included reduction of the amino proton. If this is true, one would expect that the first reduction for the free base and the hydrochloride salt might show some differences in character. Consequently, one might question the Caplis' hypothesis regarding the part played by the proton in this reduction step.

The following are some factors that must be considered in the assay of the dosage forms:

(i) Tablets and (ii) Capsules:

General Procedure #1 may be used without any exception. However, the Tetrex preparations in which the active constituent occurs as tetracycline phosphate complex have been put through additional investigations. The analysis of standard tetracycline hydrochloride solution was carried out in pH 7.75 boric acid-borax buffer as before. In order to ascertain whether there was any effect on the normal tetracycline waves caused by presence of phosphate, disodium

hydrogen phosphate in varying amounts was added to the standard solution of tetracycline hydrochloride. The polarograms showed that unless the quantity of disodium hydrogen phosphate was greatly in excess of the amount present in the pharmaceutical preparation, no effect on the waves could be observed. The same finding was true for sodium dihydrogen phosphate. In addition, the potencies of the Tetrex preparations were usually expressed as, "containing tetracycline phosphate equivalent to a known weight (eg. 100 mg, 250 mg or 500 mg) of tetracycline hydrochloride per tablet or capsule". Therefore, although no direct reliable calibration curve prepared from its standard solutions was available for the phosphate complex, the use of the calibration curve originally for tetracycline hydrochloride seemed to be acceptable. In the Tetrex-F (TC·phosphate-nystatin mixture) preparation, the effect due to the existence of nystatin has been examined and found to contribute some current under the conditions specified. The tetracycline phosphate content could be calculated after the blank determination for nystatin has been done.

(iii) Injection Powders in Vials:

There are two classes to be considered, (a) Those intended for IM purposes which consist of tetracycline HCl (or oxytetracycline HCl), procaine HCl, magnesium chloride and ascorbic acid. (b) Those for IV purposes which are usually claimed on the label simply as a mixture of tetracycline

HCl (or oxytetracycline HCl) and ascorbic acid.

Procaine HCl even at the concentration in which it occurs in the dosage form causes serious and undesirable influences on the waves of the tetracyclines. A suitable means of overcoming this could not be found. In addition, magnesium chloride was found to have a marked effect on the two waves of the tetracyclines. Fortunately, the addition of three moles of di-sodium EDTA per mole of magnesium chloride (actually they react in 1:1 ratio) eliminated the interference caused by the magnesium ion. This chelation was very helpful since di-sodium EDTA itself exhibited no reduction in the vicinity of the tetracycline waves.

Ascorbic acid has a distinct wave and a marked limiting current that overlaps with the first wave of the antibiotics in the boric acid-borax buffer solution at either pH 7.75 or 8.20.

It was expected to be very easy to assay tetracycline antibiotics in those preparations for IV use. However, when the polarograms of those were determined, an unexpected large maximum and one extra wave could be observed. In other words, the shape of the polarograms were significantly different from those of tetracycline and oxytetracycline salts even considering the wave due to the ascorbic acid. It might be concluded that some other polarographically active material(s) existed in those preparations.

Under the assumption that all of the components

in the dosage form were present exactly according to the label declaration, a polarogram was run on a precise mixture of all known components except the particular tetracycline. In this way, it was hoped to eliminate any contribution by them to the current of the antibiotic. Naturally, this was also under the assumption that there was no interaction among the constituents. The total diffusion current of the mixture must be equal to the algebric sums of those of the individual components.

(iv) Ointments:

Achromycin Ointment and Aureomycin Ointment were prepared respectively by incorporating the hydrochloride salts of tetracycline or chlortetracycline in a mixture of petrolatum and liquid petrolatum. It has been mentioned earlier that the hydrochloride salts of these two antibiotics are quite water soluble. In considering the partition coefficients of these drugs between water and the petrolatum, it was found advisable to extract them several times with the specified buffer solutions. The intensity of the inherent yellow color due to the antibiotic gradually decreased indicating that the extraction was approaching completion. However, this criterion was useful only when white petrolatum was employed as the base. If the base of the ointment was yellow petrolatum or otherwise intensely colored, TLC techniques or some other specific spot test might have been applied to investigate the progress of

extraction. The main purpose of this examination was to ascertain the minimum number of times it was necessary to extract the drug in order to ensure complete recovery.

Problems were encountered in the assay of Terramycin Ophthalmic Ointment and Terramycin Topical Ointment both of which are mixtures of oxytetracycline hydrochloride and polymyxin B sulfate in a base of white petrolatum and liquid petrolatum. Both these two antibiotics are principle ingredients. Each is quite water soluble and both give polarographic waves which interfere with each other. This method of assay is based on the linear relationship between the total current and the concentration of oxytetracycline hydrochloride solution. Unfortunately, the polymyxin B sulfate wave gave a very sharp rise in the region where the total current of oxytetracycline hydrochloride is normally measured. Therefore, further work on the analysis of these products was abandoned until a complete separation or polarographic inactivation of either one of these two antibiotics had been accomplished. This was not undertaken as part of this study.

(v) Syrups:

Since the tetracyclines in these dosage forms existed either as the free base (Achromycin) or as an insoluble complex form (Terramycin), the addition of an appropriate amount of 3 M hydrochloric acid was necessary to bring them into solution. The Terramycin Syrup, in which the oxytetra-

cycline existed as a calcium-oxytetracycline complex, needed further treatment, because the addition of hydrochloric acid split the complex to produce oxytetracycline hydrochloride and calcium chloride (or Ca^{++}). The latter product had to be sequestered or inactivated by further addition of di-sodium EDTA. Because of the addition of hydrochloric acid and di-sodium EDTA, a careful adjustment of pH of the solution was accomplished by adding an appropriate amount of borax powder.

(vi) Suspensions:

The tetracyclines in these pharmaceutical preparations also existed as the free bases. Consequently, the addition of an appropriate amount of 3 M hydrochloric acid was required and it was also necessary to adjust the pH by the careful addition of borax. General Procedure #2 then could be followed directly.

(vii) Pediatric Drops:

In this preparation the oxytetracycline existed as a calcium-dioxytetracycline complex which was not soluble in water. But when the modified procedures were applied as previously outlined for Terramycin Syrup, a typical polarogram then could be obtained.

Where possible, the assay results of the proposed method have been compared with those obtained by the manufacturers using their methods for the individual dosage

Table X Results of DC Polarographic Assay for Tetracycline Antibiotics

Commercial Product	Manufacturer	Labelled Drug Content	% Labelled Strength	
			Polaro-graphic	Manufacturer's Result
Tetracyn Capsule	Pfizer	250 mg TC·HCl per capsule	108.88	Not Available
T-Caps	Empire Lab.	250 mg TC·HCl per capsule	107.16	112.0
Achromycin V Capsule	Lederle	250 mg TC·HCl per capsule	107.40	109.0 (a) 107.5 (b)
Tetralean Capsule	M.T.C.	250 mg TC·HCl per capsule	101.27	103.2 (a)
Novotetra 250 mg Capsule	Novopharm	250 mg TC·HCl per capsule	99.89	98.2 (a) 101.0 (b)
Achromycin IM Injection Powder	Lederle	100 mg TC·HCl per vial	unable to assay	105.0 (a)
Tetracyn IM Injection Powder	Pfizer	250 mg TC·HCl per vial	unable to assay	Not Available

TC is tetracycline.

(a) microbiological assay.

(b) chemical assay.

Table X continued .....

Commercial Product	Manufacturer	Labelled Drug Content	% Labelled Strength	
			Polaro-graphic	Manufacturer's Result
Tetracyn IV Injection Powder	Pfizer	500 mg TC·HCl per vial	uneble to assay	Not Available
T-Tabs	Empire Lab.	250 mg TC·HCl per tablet	104.16	106.0 (a)
Novotetra Tablet	Novopharm	250 mg TC·HCl per tablet	96.67	102.5 (a) 97.0 (b)
Achromycin Eye-Ear Ointment	Cyanamid	1% TC·HCl in 1/8 oz ointment	110.20	106.6 (a) 115.8 (b)
Achromycin Topical Ointment	Cyanamid	3% TC·HCl in 30 gm ointment	108.80	103.5 (c)
Novotetra Suspension	Novopharm	containing TC equi-valent to 125 mg TC·HCl per 5 ml	134.67	Not Available
Tetrex Syrup	Bristol	25 mg TC·HCl per ml	107.34	112.8 (a)

TC is tetracycline.

(a) microbiological assay.

(b) chemical assay.

(c) chromatographic assay.

Table X continued .....

Commercial Product	Manufacturer	Labelled Drug Content	% Labelled Strength	
			Polarographic	Manufacturer's Result
Achromycin Syrup	Lederle	25 mg TC per ml	110.74	102.9 (a) 116.0 (b)
Tetracyn Oral Suspension	Pfizer	containing TC equivalent to 25 mg TC·HCl per ml	124.45	Not Available
T-Liquid	Empire Lab.	containing TC equivalent to 25 mg TC·HCl per ml	134.59	113.1
Aureomycin Topical Ointment	Lederle	3% CTC·HCl in 30 gm ointment	100.73	107.8 (a) 112.8 (b)
Aureomycin Ophthalmic Ointment	Lederle	1% CTC·HCl in 1/8 oz ointment	89.61	94.8 (a) 106.8 (b)
Aureomycin Capsule	Lederle	250 mg CTC·HCl per capsule	106.72	108.0 (a) 108.8 (b)

TC is tetracycline.

CTC is chlortetracycline.

(a) microbiological assay.

(b) chemical assay.

Table X continued .....

Commercial Product	Manufacturer	Labelled Drug Content	% Labelled Strength	
			Polaro-graphic	Manufacturer's Result
Declomycin 300 Tablet	Lederle	300 mg DMCTC·HCl per tablet	106.38	110.0 (a) 103.7 (b)
Declomycin Capsule	Lederle	150 mg DMCTC·HCl per capsule	104.83	110.1 (a) 105.9 (b)
Tetrex Capsule 100 mg	Bristol	containing TC·phosphate = 100 mg TC·HCl per capsule	109.59	108.0 (a)
Tetrex Capsule 250 mg	Bristol	containing TC·phosphate = 250 mg TC·HCl per capsule	102.11	106.4 (a)
Tetrex Bid Caps 500 mg	Bristol	containing TC·phosphate = 500 mg TC·HCl per capsule	100.79	102.0 (a)

TC is tetracycline and DMCTC is demethylchlortetracycline.

(a) microbiological assay.

(b) chemical assay.

Table X continued .....

Commercial Product	Manufacturer	Labelled Drug Content	% Labelled Strength	
			Polaro-graphic	Manufacturer's Result
Tetrex-F Capsule	Bristol	containing TC·phosphate & Nystatin, TC·phosphate = 250 mg TC·HCl per capsule	100.17	108.8 (a)
Terramycin IM Solution	Pfizer	containing OTC.HCl = 250 mg OTC per ampoule	unable to assay	114.2 (a)
Terramycin Ophthalmic Ointment	Pfizer	each gm containing OTC·HCl = 5 mg OTC and 10,000 units of Polymyxin B sulfate	unable to assay	OTC·HCl 100.0 Polymyxin B sulfate 102.5 (a)
Terramycin Topical Ointment	Pfizer	each gm containing OTC·HCl = 30 mg OTC and 10,000 units of Polymyxin B sulfate	unable to assay	OTC·HCl 106.0 (a)
Terramycin Syrup	Pfizer	each ml containing Ca·OTC = 25 mg OTC	127.19	114.0 (a)

TC is tetracycline, and OTC is oxytetracycline.
(a) microbiological assay.

Table X continued .....

Commercial Product	Manufacturer	Labelled Drug Content	% Labelled Strength	
			Polaro-graphic	Manufacturer's Result
Terramycin Pedia-tric Drop	Pfizer	each ml containing Ca·di-OTC = 100 mg OTC	96.48	113.0
Terramycin Capsule	Pfizer	containing OTC·HCl = 250 mg OTC per capsule	103.42	103.93
Terramycin IV Injection Powder	Pfizer	each vial containing OTC·HCl = 500 mg OTC buffered with 2.0 gm ascorbic acid	unable to assay	102.38
Novoxytetra Capsule	Novopharm	250 mg OTC·HCl per capsule	98.31	99.4 (a) 98.3 (b)

OTC is oxytetracycline.

(a) microbiological assay.

(b) chemical assay.

forms. From an examination of Table X, it can be seen that the values obtained by the polarographic method agree favorably with the manufacturers' results for all tablets and capsules included in this investigation. Some discrepancies in the results obtained by the two methods can be seen for certain ointment preparations. This can be generally attributed to the degree of extraction and also possibly due to some chemical alteration and degradation of the drug upon heating. Some significant variations can be found for certain liquid preparations including syrups, suspensions and aqueous drops. This can be rationalized by the fact that in most of the cases, the preservatives, solubilizers and stabilizers for the liquid preparations are not stated on the labels and yet they may exhibit polarographic reduction. Furthermore, the expiry date shown on the label of each preparation indicates the time when the drug has reached the minimum allowable potency level. A small degree of chemical alteration and degradation of the constituents of the preparations can be expected between the time of the manufacturer's assay immediately after its production and that of the present polarographic assay. The rate of chemical alteration of the tetracyclines is much more rapid when they are present in liquid formulations, particularly in an aqueous medium, than when they are present in dry, solid dosage forms such as injection powders, tablets and capsules. In most of the pharma-

ceutical manufacturing laboratories, the weight variation allowed in quality control level is around ±5% of its mean value, so that it is very possible to have some difference of mean weight due to sampling error.

Under those conditions where the preservatives, solubilizers, stabilizers, filling materials and adjuvants are known, a blank determination could be performed and the true current contribution of the tetracyclines thus could be accurately obtained. Except in a few instances where the separation from interfering material(s) has to be achieved, this polarographic method would seem to have a relatively high degree of applicability and is readily applicable to the assays of a large variety of dosage forms.

J. Quantitative Analysis of Tetracyclines in Blood and Urine

Interest has been extended to the polarographic estimation of tetracyclines in blood and urine. Most of the studies were aimed at an extraction technique which could supply a definite recovery rate. The procedures devised by Scales et al.(69) have been modified and applied to the estimation in blood. However, due to the relatively low blood level of tetracyclines, efficiency of extraction and the sensitivity limitation of DC polarography, a large volume (at least 100 mls) of blood would be required for a single determination. Obviously this is not clinically feasible. The tetracycline levels in urine reported

in the literature were about 1 mg/21-25 ml, therefore, it was possible and meaningful to do further investigation in urine. In short, those procedures described in the Experimental Section involved mainly protein precipitation and solvent extraction techniques.

The simple evaporation of the solution of oxytetracycline in ethylacetate, for example, was sufficient to destroy all of the oxytetracycline. This effect was presumably due in part to photodecomposition since, when the evaporation was carried out in darkness, about 20-45% recovery was usually obtained. The addition of the anti-oxidant, β-mercaptopropionic acid, in various stages could result in 98-100% recovery of oxytetracycline from the solvent, however, it also complicated the polarographic determination, because β-mercaptopropionic acid could give irregular waves under the conditions which were optimum for oxytetracycline.

If the volume of solution in the polarographic cell was reduced, this might allow one to use a smaller volume of urine sample and reagents, but the Comparative Method in polarography requires identical conditions for both standards and samples. Therefore, a new calibration curve would have to be established.

β-Mercaptopropionic acid could not be used as an anti-oxidant in any stage of the extraction procedure. However, a simple extraction and evaporation of the solvent in subdued light gave low and variable recoveries, conse-

quently a new method must be devised. Such an investigation is beyond the scope of this study.

K. Mechanism of Electroreduction

In his studies of the electroreduction mechanism of tetracycline, Caplis (62) has investigated polarograms both in aqueous and non-aqueous systems and finally postulated that the electroreduction involved the proton on the amino group and the C_1 carbonyl system in the A ring of the tetracycline molecule. This postulation has been considered in the study of the systems which were employed in the present investigation. For practical purposes of analysis, Meites (77) has expressed the equation of the wave which involves a single rate determining step at 25°C as:

$$E_{applied} = E_{1/2} + \frac{0.0591}{\alpha n} \log \frac{i_d - i}{i} \quad \cdots\cdot \text{(Equation 6)},$$

where

$E_{applied}$ is the applied potential,

$E_{1/2}$ is the half-wave potential.

α is the transfer coefficient (the fraction of the potential change that increases the rate constant of the cathodic process).

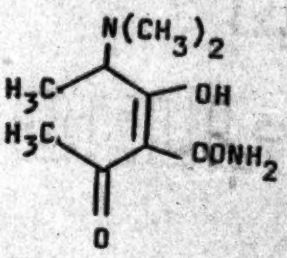
n is the number of electron(s) involved in the rate determining electrochemical step (activation step).

i and i_d are average current at any point (but here the

residual current, i_r, has been deducted) and average diffusion current at that point respectively.

Theoretically, for the irreversible wave with a single rate determining step, the plot of applied potential, $E_{applied}$, against log $(i_d - i)/ i$ should be linear with a slope of $0.0591/\alpha n$. However, when the polarographic measurements and the successive calculations were performed for tetracycline hydrochloride, the plot of applied potential versus log $(i_d - i)/ i$ gave several segments of straight line each having a different slope. This situation occurred whether the two waves were analyzed separately or as one continuous polarogram. The interpretation of the plots was difficult and, in some instances, the n values obtained had little or no significance (see Table XI, Figure 22 and Table XII, Figure 23). This fact was common to oxytetracycline hydrochloride, chlortetracycline hydrochloride and demethylchlortetracycline hydrochloride. Since no clear indication of the number of electrons involved in the process was obtained, it confirmed more definitely that the mechanism of reduction for these substances was very complex. In some cases, the sharp slopes might be due to transfer coefficients which were much less than one. To attempt to clarify the electroreduction mechanism, it may be helpful to determine their electron spin resonance spectra to see which carbonyl group is reduced or whether some other group is involved. Another useful experiment may be based on the changes of the IR

spectra of tetracyclines before and after electroreduction, especially at around 1700 cm^{-1} region. The third possibility is to get a comparison of the NMR spectra before and after electroreduction using D_2O (or $\begin{matrix}CD_3\\CD_3\end{matrix}\!\!>\!C=O$ or C_2H_5OD) as solvent, because the peak assignment may be especially useful. Finally, the UV spectra resulting from the conjugation of the A ring of the tetracyclines (12) could be compared with the spectra of the reduced compound. If the C_1 carbonyl is involved in the reduction, this should be evident by a shift in the absorption wavelength, similar to the reduction from



to

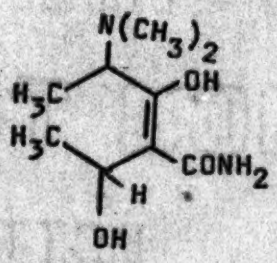


Table XI Data for Logarithmic Analysis of Current-Voltage Curve of Tetracycline Hydrochloride (1.56×10^{-4}M) Here the Two Waves Were Studied Continuously.

Applied Potential vs SCE	$(i_d - i)/ i$	log $(i_d - i)/i$
-1.00	149.0- 18.7/ 18.7-17.8	2.1607
-1.02	149.1- 19.1/ 19.1-17.9	2.0346
-1.04	149.5- 20.0/ 20.0-18.1	1.8335
-1.08	150.1- 23.0/ 23.0-18.5	1.4509
-1.10	150.5- 26.0/ 26.0-18.9	1.2439
-1.14	151.1- 34.0/ 34.0-19.3	0.9013
-1.18	151.8- 44.4/ 44.4-19.9	0.6419
-1.20	152.1- 50.0/ 50.0-20.0	0.5318
-1.24	152.9- 60.3/ 60.3-20.3	0.3645
-1.28	153.3- 68.3/ 68.3-21.0	0.2546
-1.30	153.8- 72.2/ 72.2-21.1	0.2033
-1.34	154.2- 81.0/ 81.0-21.7	0.0914
-1.38	155.0- 90.6/ 90.6-22.0	-0.0274
-1.40	155.2- 96.3/ 96.3-22.2	-0.0997
-1.44	155.9-109.2/109.2-22.9	-0.2667
-1.48	156.5-126.5/126.5-23.2	-0.5370
-1.50	156.9-132.8/132.8-23.4	-0.6570
-1.54	157.3-142.3/142.3-24.0	-0.8968
-1.58	158.0-149.0/149.0-24.4	-1.1413
-1.60	158.2-152.0/152.0-24.8	-1.3121

Table XI: continued .....

Applied Potential vs SCE	$(i_d - i)/ i$	$\log (i_d - i)/ i$
-1.62	158.6-154.1/154.1-25.0	-1.457[illegible]
-1.64	158.9-156.0/156.0-25.1	-1.6544
-1.68	159.5-158.7/158.7-25.6	-2.2211

FIGURE 22:

LOGARITHMIC ANALYSIS OF CURRENT-VOLTAGE CURVE TETRACYCLINE HYDROCHLORIDE AT CONCENTRATION OF 1.56×10^{-4} M AND TEMPERATURE OF 25.0° C (THE TWO WAVES STUDIED CONTINUOUSLY)

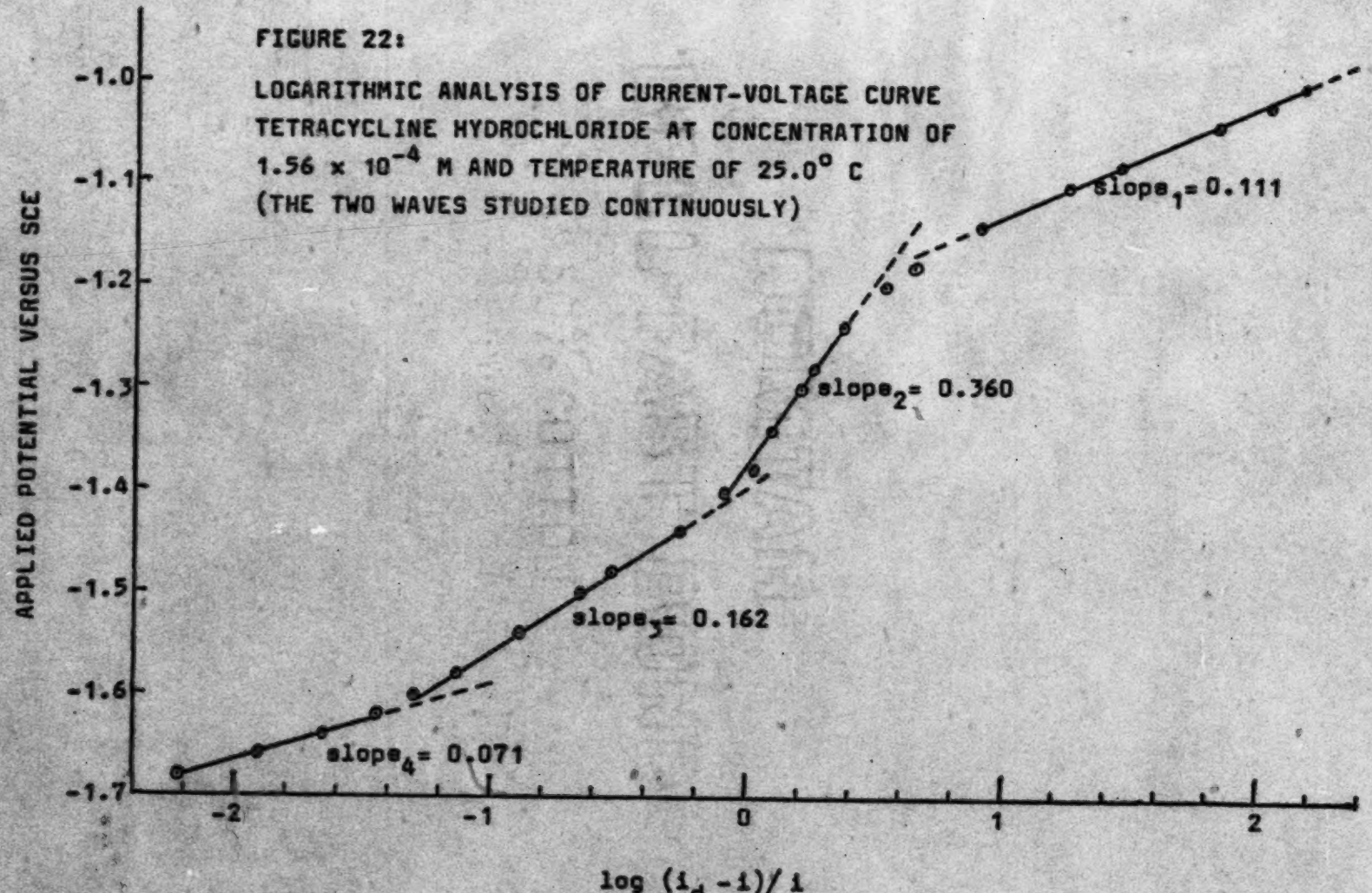


Table XII Data for Logarithmic Analysis of Current-Voltage Curve of Tetracycline Hydrochloride(1.56×10^{-4}M) Here the Two Waves Were Studied Separately.

Applied Potential vs SCE	$(i_d - i)/ i$	$\log (i_d - i)/i$
-1.00	59.8- 16.4/ 16.4-15.5	1.6832
-1.02	60.0- 17.0/ 17.0-15.9	1.5921
-1.04	60.2- 17.6/ 17.6-16.0	1.4253
-1.06	60.5- 18.6/ 18.6-16.2	1.2419
-1.08	60.9- 20.9/ 20.9-16.6	0.9685
-1.10	61.0- 23.2/ 23.2-16.9	0.7782
-1.12	61.2- 26.2/ 26.2-17.1	0.5851
-1.14	61.6- 30.0/ 30.0-17.3	0.3959
-1.16	61.9- 33.7/ 33.7-17.8	0.2488
-1.18	62.1- 38.0/ 38.0-18.0	0.0809
-1.20	62.3- 41.9/ 41.9-18.1	-0.0669
-1.22	62.8- 45.2/ 45.2-18.5	-0.1810
-1.24	63.0- 48.2/ 48.2-18.8	-0.2981
-1.26	63.2- 51.0/ 51.0-19.0	-0.4188
-1.28	63.5- 53.2/ 53.2-19.2	-0.5187
-1.30	63.8- 55.9/ 55.9-19.5	-0.6635
-1.32	64.0- 59.0/ 59.0-19.9	-0.8931
-1.34	64.2- 61.7/ 61.7-20.0	-1.2222
-1.36	- - - - - - -	- -
-1.38	108.4- 68.4/ 68.4-64.9	1.0580

Table XII: continued . . . . .

Applied Potential vs SCE		
-1.40	108.8- 73.0/ 73.0-65.1	0.6563
-1.42	109.0- 80.0/ 80.0-65.6	0.3040
-1.44	109.3- 86.0/ 86.0-66.0	0.0664
-1.46	109.8- 91.9/ 91.9-66.1	-0.1587
-1.48	110.0- 96.2/ 96.2-66.5	-0.3329
-1.50	110.1- 99.5/ 99.5-66.8	-0.4892
-1.52	110.4-102.5/102.5-67.0	-0.6536
-1.54	110.8-105.3/105.3-67.2	-0.8405
-1.56	111.0-107.2/107.2-67.5	-1.0190
-1.58	111.1-109.4/109.4-67.9	-1.3876
-1.60	111.4-110.7/110.7-68.0	-1.7853

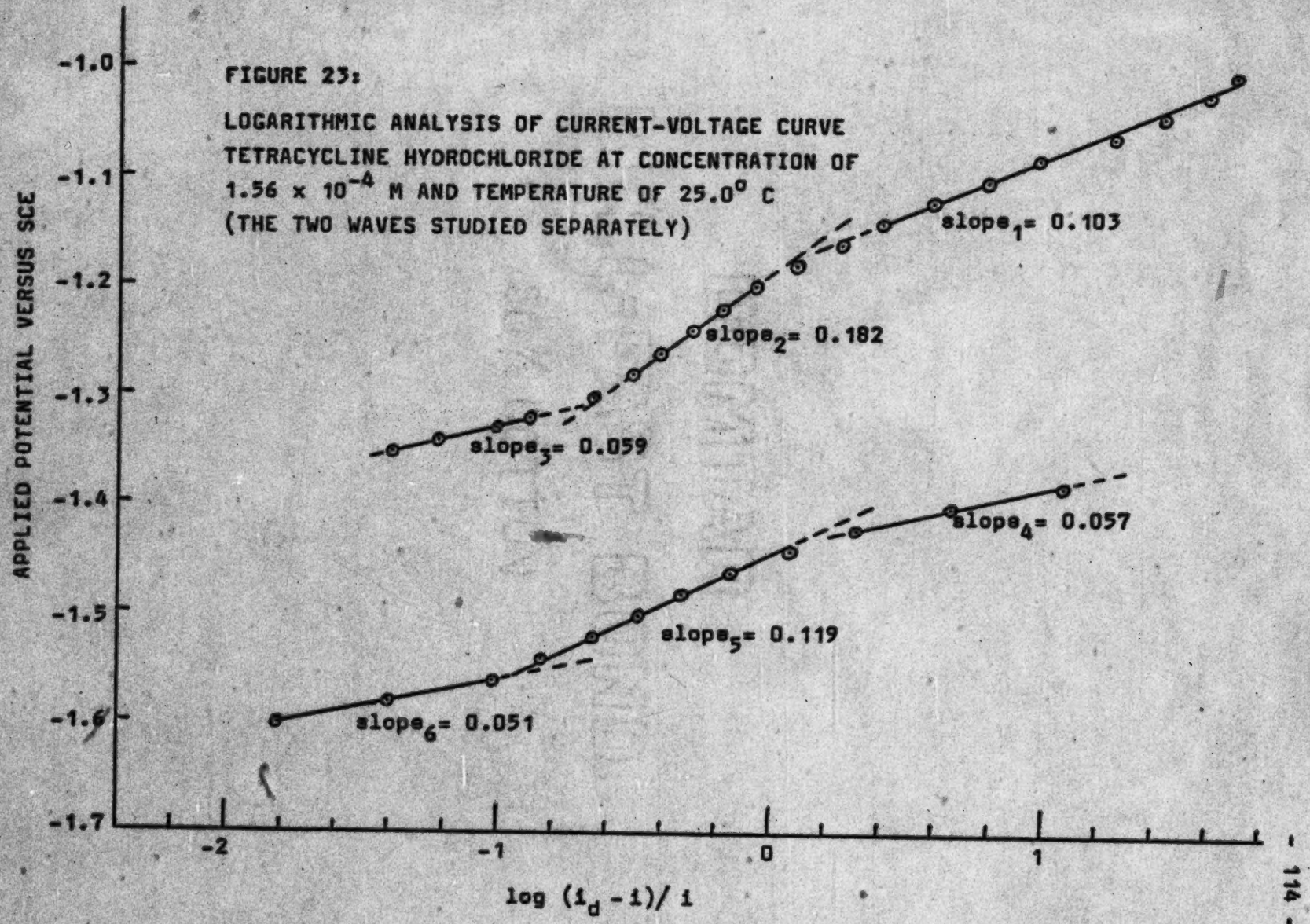


FIGURE 23:
LOGARITHMIC ANALYSIS OF CURRENT-VOLTAGE CURVE TETRACYCLINE HYDROCHLORIDE AT CONCENTRATION OF 1.56 x 10^-4 M AND TEMPERATURE OF 25.0° C (THE TWO WAVES STUDIED SEPARATELY)
APPLIED POTENTIAL VERSUS SCE
-1.0
-1.1
-1.2
-1.3
-1.4
-1.5
-1.6
-1.7
-2
-1
0
1
log (i_d - i)/ i
slope_1= 0.103
slope_2= 0.182
slope_3= 0.059
slope_4= 0.057
slope_5= 0.119
slope_6= 0.051

SUMMARY AND CONCLUSIONS

(1) A sensitive DC polarographic method of analysis for the four most popular members of the tetracycline series and their pharmaceutical dosage forms has been developed which is based on the electroreduction of certain functional groups of the antibiotics. This technique was applied to various pharmaceutical dosage forms and favorably agreeable results were obtained for most of the cases.

(2) The polarographic determination of tetracyclines in urine might be applicable when an efficient and definite extraction procedure is developed.

(3) The actual mechanism of the electroreduction has not been postulated as the process is obviously very complicated. It may involve the conjugated carbonyl system in the A ring of the molecules as well as other functional groups in the tetracyclines. However, further examination of UV, visible, IR and NMR spectra before and after electroreduction might be useful. The investigation of electron spin resonance spectra might also be useful in this case.

(4) Studies of the effects of the composition of supporting electrolyte, the effects of pH of the system and of heights of the mercury column have been conducted. The electroreduction has been confirmed mainly as diffusion dependent in the ranges of mercury column

height and concentration selected. From the studies it was found that the mercury column height of 65.0 cm was suitable in each instance. A boric acid-borax buffer was selected as being the most suitable system for the electroreduction of all of the tetracyclines in this investigation. All measurements were made by employing a saturated calomel electrode (SCE) as the reference electrode. The following special conditions were required for each antibiotic:

(a) tetracycline HCl, a pH of 7.75 was required and the total current was measured at -1.70 V.

(b) oxytetracycline HCl, the pH was 8.20 and the applied potential was -1.60 V.

(c) chlortetracycline HCl, the pH was 7.95 and the applied potential was -1.66 V.

(d) demethylchlortetracycline HCl, the pH was 7.75 and the applied potential was -1.70 V. It was necessary, however, to add a carefully measured amount of freshly prepared 1% gelatin solution in order to eliminate a maximum in the wave of this antibiotic. The final concentration of the maximum suppressor was 0.005% w/v.

(5) Linear and reproducible calibration curves for the four tetracyclines were obtained using pure reference materials supplied by the manufacturers. It was not necessary to prepare an additional calibration curve

with pure reference tetracycline phosphate, since the calibration curve for tetracycline hydrochloride could be readily utilized.

(6) The DC polarographic determination was based on a linear relationship plot between the total current and the concentration of the species. The electroreduction was not necessarily specific for the tetracyclines alone, therefore, the performance of a blank determination was required. In some instances, a prior separation from interfering material(s) was essential.

(7) It is concluded that the accuracy and reproducibility of this DC polarographic method make it favorably acceptable for the analysis of tetracyclines. The technique offers a greater degree of sensitivity than most of the existing chemical methods and, in addition, offers a specificity that is unknown in the microbiological method.

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APPENDIX

Table XIII Data for Analysis of Pharmaceutical Dosage Forms

Commercial Product	Manufacturer	Theoretical Final Conc. in mg/10 ml	Total Current in μA	Found and Corrected Final Conc. in mg/10 ml	% Labelled Strength	Average
Tetracyn Capsule 250 mg TC·HCl per capsule	Pfizer	0.771	2.2320	0.844	109.47	108.88 ± 0.63
		0.774	2.2290	0.842	108.79	
		0.792	2.2650	0.858	108.33	
		0.793	2.2995	0.869	109.58	
		0.791	2.2620	0.856	108.22	
T-Caps 250 mg TC·HCl per capsule	Empire Lab.	0.761	2.1660	0.818	107.49	107.16 ± 0.48
		0.771	2.1735	0.821	106.49	
		0.780	2.2185	0.839	107.56	
		0.780	2.2035	0.833	106.79	
		0.779	2.2110	0.837	107.45	
Achromycin V Cap. 250 mg TC·HCl per capsule	Lederle	0.775	2.2170	0.838	108.13	107.40 ± 0.73
		0.777	2.2200	0.840	108.11	
		0.786	2.2335	0.844	107.38	
		0.787	2.2163	0.838	106.48	
		0.795	2.2425	0.850	106.92	
Tetralean Capsule 250 mg TC·HCl per capsule	M.T.C.	0.784	2.1240	0.794	101.28	101.27 ± 0.45
		0.784	2.1090	0.789	100.64	
		0.785	2.1270	0.795	101.27	
		0.787	2.1435	0.802	101.91	
		0.786	2.1323	0.796	101.27	

TC is tetracycline.

Table XIII continued ....

Commercial Product	Manufacturer	Theoretical Final Conc. in mg/10 ml	Total Current in μA	Found and Corrected Final Conc. in mg/10 ml	% Labelled Strength	Average
Novotetra 250 mg Capsule 250 mg TC·HCl per capsule	Novopharm	0.766	2.0490	0.764	99.74	99.89 ± 0.99
		0.766	2.0625	0.770	100.52	
		0.766	2.0220	0.754	98.43	
		0.766	2.0760	0.774	101.04	
		0.766	2.0490	0.764	99.74	
T-Tabs 250 mg TC·HCl per tablet	Empire Lab.	0.553	1.5660	0.576	104.16	104.16 ± 0.23
		0.543	1.5465	0.567	104.42	
		0.552	1.5645	0.575	104.17	
		0.543	1.5450	0.566	104.26	
		0.552	1.5600	0.573	103.80	
Novotetra Tablet 250 mg TC·HCl per tablet	Novopharm	0.751	1.9425	0.721	96.01	96.67 ± 0.54
		0.751	1.9440	0.724	96.40	
		0.751	1.9680	0.732	97.47	
		0.751	1.9500	0.727	96.80	
		0.751	1.9470	0.726	96.67	
Achromycin Eye-Ear Ointment 1% TC·HCl in 1/8 oz ointment	Cyanamid	0.751	2.2110	0.828	110.25	110.20 ± 0.15
		0.751	2.2095	0.827	110.12	
		0.751	2.2110	0.828	110.25	
		0.751	2.2080	0.826	109.99	
		0.751	2.2155	0.829	110.39	

TC is tetracycline.

Table XIII continued .....

Commercial Product	Manufacturer	Theoretical Final Conc. in mg/10 ml	Total Current in μA	Found and Corrected Final Conc. in mg/10 ml	% Labelled Strength	Average
Achromycin Topical Ointment 3% TC·HCl in 30 gm ointment	Cyanamid	0.593	1.7430	0.644	108.60	108.80 ± 0.19
		0.593	1.7450	0.645	108.77	
		0.593	1.7500	0.647	109.11	
		0.593	1.7450	0.645	108.77	
		0.593	1.7440	0.645	108.77	
Novotetra Suspension containing TC = 125 mg TC·HCl per 5 ml	Novopharm	0.750	2.6835	1.013	135.07	134.67 ± 0.89
		0.750	2.6850	1.013	135.07	
		0.750	2.6850	1.013	135.07	
		0.750	2.6850	1.013	135.07	
		0.750	2.6445	0.998	133.07	
Tetrex Syrup 25 mg TC·HCl per ml	Bristol	0.750	2.1480	0.803	107.07	107.34 ± 0.41
		0.750	2.1495	0.803	107.07	
		0.750	2.1483	0.803	107.07	
		0.750	2.1645	0.810	108.00	
		0.750	2.1540	0.80[illegible]	107.47	
Achromycin Syrup 25 mg TC per ml	Lederle	0.812	2.4015	0.903	111.21	110.74 ± 0.51
		0.812	2.4030	0.903	111.21	
		0.812	2.3940	0.899	110.71	
		0.812	2.3790	0.893	109.98	
		0.812	2.3895	0.898	110.59	

TC is tetracycline.

Table XIII continued .....

Commercial Product	Manufacturer	Theoretical Final Conc. in mg/10 ml	Total Current in μA	Found and Corrected Final Conc. in mg/10 ml	% Labelled Strength	Average
Tetracyn Oral Suspension containing TC = 25 mg TC·HCl per ml	Pfizer	0.750	2.4855	0.935	124.67	124.45 ± 0.24
		0.750	2.4840	0.934	124.53	
		0.750	2.4810	0.931	124.13	
		0.750	2.4825	0.932	124.27	
		0.750	2.4855	0.935	124.67	
T-Liquid containing TC = 25 mg TC·HCl per ml	Empire Lab.	0.750	2.6775	1.010	134.67	134.59 ± 0.08
		0.750	2.6760	1.009	134.53	
		0.750	2.6775	1.010	134.67	
		0.750	2.6760	1.009	134.53	
		0.750	2.6745	1.009	134.53	
Aureomycin Topical Ointment 3% CTC·HCl in 30 gm ointment	Lederle	0.656	1.6673	0.672	102.44	100.73 ± 1.10
		0.656	1.6395	0.657	100.15	
		0.656	1.6388	0.656	100.00	
		0.656	1.6350	0.655	99.85	
		0.656	1.6515	0.664	101.22	
Aureomycin Ophthalmic Ointment 1% CTC·HCl in 1/8 oz ointment	Lederle	0.660	1.4918	0.592	89.70	89.61 ± 0.44
		0.660	1.4790	0.587	88.94	
		0.660	1.4955	0.595	90.15	
		0.660	1.4895	0.592	89.70	
		0.660	1.4865	0.591	89.55	

TC is tetracycline.

CTC is chlortetracycline.

Table XIII continued .....

Commercial Product	Manufacturer	Theoretical Final Conc. in mg/10 ml	Total Current in μA	Found and Corrected Final Conc. in mg/10 ml	% Labelled Strength	Average
Aureomycin Capsule 250 mg CTC·HCl per capsule	Lederle	0.708	1.8450	0.752	106.21	106.72 ± 0.83
		0.708	1.8465	0.752	106.21	
		0.708	1.8510	0.754	106.50	
		0.708	1.8660	0.766	108.19	
		0.708	1.8510	0.754	106.50	
Declomycin 300 Tablet 300 mg DMCTC·HCl per tablet	Lederle	0.642	2.1795	0.684	106.54	106.68 ± 0.22
		0.642	2.1705	0.682	106.23	
		0.642	2.1668	0.681	106.07	
		0.642	2.1795	0.684	106.54	
		0.642	2.1795	0.684	106.54	
Declomycin Capsule 150 mg DMCTC·HCl per capsule	Lederle	0.687	2.2980	0.724	105.39	104.83 ± 0.42
		0.687	2.2890	0.721	104.95	
		0.687	2.2725	0.716	104.22	
		0.676	2.2545	0.709	104.88	
		0.676	2.2485	0.708	104.73	
Tetrex Capsule containing TC·phosphate = 100 mg TC·HCl per capsule	Bristol	0.680	1.9950	0.742	109.12	109.59 ± 0.26
		0.680	2.0025	0.746	109.71	
		0.680	2.0025	0.746	109.71	
		0.680	2.0025	0.746	109.71	
		0.680	2.0025	0.746	109.71	

CTC is chlortetracycline, DMCTC is demethylchlortetracycline, and TC is tetracycline.

Table XIII continued .....

Commercial Product	Manufacturer	Theoretical Final Conc. in mg/10 ml	Total Current in μA	Found and Corrected Final Conc. in mg/10 ml	% Labelled Strength	Average
Tetrex Capsule 250 mg containing TC·phosphate= 250 mg TC·HCl per capsule	Bristol	0.748	2.0475	0.764	102.14	102.11 ± 0.14
		0.748	2.0520	0.765	102.27	
		0.748	2.0475	0.764	102.14	
		0.748	2.0415	0.762	101.87	
		0.748	2.0475	0.764	102.14	
Tetrex Bid Caps 500 mg containing TC·phosphate= 500 mg TC·HCl per capsule	Bristol	0.760	2.0490	0.764	100.53	100.79 ± 0.82
		0.760	2.0475	0.764	100.53	
		0.760	2.0415	0.762	100.26	
		0.760	2.0835	0.777	102.24	
		0.760	2.0460	0.763	100.39	
Tetrex-F Capsule containing TC·phosphate= 250 mg TC·HCl, and Nystatin	Bristol	0.727	1.9620	0.729	100.28	100.17 ± 0.18
		0.727	1.9605	0.728	100.14	
		0.727	1.9620	0.729	100.28	
		0.727	1.9635	0.729	100.28	
		0.727	1.9575	0.726	99.86	
Terramycin Syrup each ml containing Ca·OTC = 25 mg OTC	Pfizer	0.809	2.1750	1.025	126.70	127.19 ± 0.73
		0.809	2.1915	1.032	127.56	
		0.809	2.1645	1.021	126.21	
		0.809	2.1900	1.031	127.44	
		0.809	2.1990	1.036	128.06	

TC is tetracycline.

OTC is oxytetracycline.

Table XIII continued .....

Commercial Product	Manufacturer	Theoretical Final Conc. in mg/10 ml	Total Current in μA	Found and Corrected Final Conc. in mg/10 ml	% Labelled Strength	Average
Terramycin Pediatric Drop each ml containing Ca-di-OTC= 100 mg OTC	Pfizer	0.863	1.7850	0.834	96.64	96.48 ± 0.36
		0.863	1.7745	0.828	95.94	
		0.863	1.7910	0.836	96.87	
		0.863	1.7798	0.831	96.29	
		0.863	1.7820	0.834	96.64	
Terramycin Capsule containing OTC·HCl = 250 mg OTC per capsule	Pfizer	0.707	1.5645	0.726	102.69	103.42 ± 0.82
		0.707	1.5825	0.736	104.10	
		0.707	1.5870	0.738	104.38	
		0.707	1.5600	0.725	102.55	
		0.707	1.5735	0.731	103.39	
Novoxytetra Capsule 250 mg OTC·HCl per capsule	Novopharm	0.779	1.6500	0.768	98.59	98.31 ± 0.25
		0.779	1.6425	0.765	98.20	
		0.760	1.6035	0.746	98.16	
		0.760	1.6020	0.745	98.03	
		0.760	1.6155	0.749	98.55	

OTC is oxytetracycline.